Molecular diagnostics for public and environmental health

Rice, Jack

Award date:
2020

Awarding institution:
University of Bath

Molecular diagnostics for public and environmental health

Volume 1 of 1

Jack Rice

A thesis submitted for the degree of Doctor of Philosophy

University of Bath

Department of Chemistry

Faculty of Science

September 2019

Thesis introduction

Copyright notice

Restrictions on use and licensing

Access to this thesis in print or electronically is restricted until:

Signed on behalf of the Doctoral College:

Declaration of any previous submission of the work

The material presented here for examination for the award of a higher degree by research has been incorporated into a submission for another degree. Work presented in chapter two in the paper *Stereochemistry of ephedrine and its environmental significance: Exposure and effects directed approach* concerning the use of river water microcosms and ephedrine toxicity to *D. Magna* was used for the completion of the candidate's final year masters projected completed May 2014 submitted to the University of Bath, where the candidate was awarded the degree Master of Chemistry in June 2014.

Candidate's signature: 

Declaration of authorship

I am the author of this thesis, and the work described therein was carried out by myself personally, with the exceptions of chapters two and three where work was carried out in collaboration with other researchers as described by the statement of authorship preceding each chapter.

Candidate's signature: 

Table of contents

List of tables and figures

Chapter one

Chapter two

Acknowledgments

I would like to thank my supervisory team, prof. Barbara Kasprzyk-Hordern and Dr. Anneke Lubben for their confidence and support during my PhD. I'd also like to thank my co-authors for their contributions to published work, the members of the environmental chemistry group at the University of Bath, past and present, for their camaraderie and willingness to go sampling at 6am on a weekend, my friends, family and partner Josie for their love, support and willingness to proof read at all hours, and the members of the University of Bath counselling and mental health team and BANES talking therapies for their continuing support.

Summary of thesis

Abstract:

Both human and environmental health are born out of extraordinarily complex systems and no thesis could ever satisfactorily explore both, instead the focus of this document lies in both trying to get the most out of existing analytical techniques and how to apply these techniques to study new challenges. Currently, information about public health is obtained by looking at the health of a few individuals and then building this up to an understanding of the whole population such as public health England's *health survey for England* or the European Monitoring Centre for Drugs and Drug Abuse's (EMCDDA) *country drug report*. The flaws in this technique are that it relies on obtaining a representative sample of individuals in order to understand the community as a whole. Wastewater based epidemiology (WBE) is a methodology that examines influent wastewater to explore what is being consumed by the population and uses this to build up a picture of public health, relying on the assumption that wastewater is equivalent to a pooled urine sample for a population. Likewise, analysis of river water can be used to gain an understanding of environmental health by measuring the concentration of anthropogenic compounds in the environment and assessing their environmental risk. However, current assessments of human and environmental health do not allow for the collection of all available data, as collected analytical measurements are either incomplete or are lacking in context. This thesis seeks to address these limitations by exploring new analytical methodologies and techniques for collecting more comprehensive data and was focused into three main areas of research: 1) the uses of chirality in determining analyte origin and its impact on assessments of public and environmental health, 2) the development of new methodologies to maximise the information obtainable from current health biomarkers and 3) the exploration of proteins as potential new biomarkers of public health.

The importance of assessing chirality was first demonstrated in chapter one, where chiral chromatography was used to measure changes in the concentration of several drugs of abuse and human pharmaceuticals in wastewater over a period of five years. By monitoring changes in the enantiomeric fraction (EF) of chiral analytes it was possible to identify that these biomarkers originated from human consumption due to stereospecific excretion. Additionally, the EF of methamphetamine changed throughout the study, which appeared to indicate a change in how and where it was manufactured. However, the main focus of chapter one was in how WBE can be used to assess public health strategies, including addressing a need for assessing the impact of drug policy on changes in drug consumption. The novel use of local pharmaceutical prescription data alongside wastewater analysis allowed for a more thorough assessment of consumption within the population, including the observation that prescription and wastewater data showed good agreement for

painkillers that are at risk of being abused, suggesting that there was no significant illicit usage in contrast to expectations from ongoing opioid epidemics in other countries. Additionally, by comparing trends in UK drug consumption with trends in European drug consumption it was possible to identify UK specific trends, such as a rapid increase in cocaine consumption, and well as those that were European wide. Alongside this, the observation that mephedrone, a drug first banned in 2010, was no longer consumed after 2015 showed that WBE can be used to assess the efficacy of drug policy. Likewise, the identification of an increasing trend in UK drug consumption from 2016 onwards were postulated to be related to significant changes in UK drug policy that occurred in 2016. Overall, a combination of chiral analysis and local prescription data were used to verify the source of analytes in wastewater, whilst comparison with long term trends in prescription rates and European wide trends lead to the identification of a trend of increasing illicit drug use in the UK, as well as a strong correlation between prescription and wastewater concentrations of painkillers that were at risk of being abused.

In a similar vein, the importance of chiral analysis for the assessment of environmental health was explored in chapter two, where river simulating microcosms and ecotoxicity tests were used to assess the environmental risk posed by ephedrine. From microcosm testing two isomers of ephedrine were observed to decay significantly more, under environmental conditions, than the remaining isomers. Following this, the compounds with the greatest environmental persistence were also observed to be, generally, the most toxic to a range of important river organisms. River simulating microcosms also revealed that one of the less persistent and less toxic isomers of ephedrine could be converted under environmental conditions to a more persistent and toxic isomer. This observation was confirmed by a combination of retrospective data analysis and human liver microcosm experimentation to rule out other potential metabolic sources of the toxic isomer. What this showed was that it was important for environmental studies to consider individual isomers as discrete compounds when considering their environmental impact. Additionally, studies should also consider how human pharmaceuticals enter the environment, the initial composition of any isomers when they enter the environment, and how they are changed, or not, after excretion or by wastewater treatment. Chapter three showcased the utility of supercritical fluid chromatography (SFC) for developing new chiral methodologies for use in wastewater and river water for monitoring compounds of environmental concern (CECs). Initial method development was able to qualitatively analyse 140 CECs, with the final method being able to fully or semi-quantify 95 CECs and detect 75% of them in grab samples of influent wastewater, effluent wastewater or river water. Initial analysis of wastewater and river water showed that the enantiomeric fractions of several analytes changed depending on the matrix they were detected in, which highlighted the importance of assessing chirality in not just environmental samples but also in the sources of CECs. The power of SFC lies in its analytical efficiency compared to contemporary chiral liquid chromatography. By developing new chiral methods that can rapidly analyse CECs it

becomes increasingly easier for routine environmental and public health monitoring to measure chirality without sacrificing analytical power or sample throughput.

The need to explore new biomarkers was brought about by the realisation that most current WBE biomarkers are used to measure consumption, rather than directly measuring public health, although biomarkers of oxidative stress have begun to be explored. Chapter four lays out the current landscape of WBE analysis and showcases the range of biomarkers currently used, as well as an estimation of the population they've been used to study, which for drugs of abuse represented roughly 1.5 % of the total human population. The use of urinary proteomics for assessing individual health in clinical analysis was discussed and compared with how drugs of abuse were used 20 years previously before the development of WBE. The case was then made for using urinary disease proteins to assess public health via their analysis in wastewater and several potential urinary biomarkers were proposed based on a set of criteria outlined in the chapter, including: urinary excretion, a known disease-biomarker relationship and disease specificity. Chapter five then took the first tentative steps towards developing a method for the analysis of human protein biomarkers in wastewater, by developing an enzymatic digestion method for five potential biomarkers in buffer, which was then adapted to work in wastewater for the most promising two proteins. The chapter also built upon the biomarker selection criteria discussed in the previous chapter to include the selection of human specific peptides, the ability to include peptides shared with other primates as pseudo-human specific and efforts to estimate the wastewater concentration of proteins from their excreted urinary concentrations in healthy adults. Initial analysis indicated the possible detection of one peptide of the inflammation biomarker C-reactive protein (CRP) in 100 mL wastewater digests using hydrophilic interaction liquid chromatography coupled with triple quadrupole mass spectrometry. This showed that protein biomarkers of disease could be detected in wastewater using largely the same techniques as current WBE analysis, which would enable its uptake by the larger WBE community as well as facilitating direct measurements of public health.

Overview and aims:

Broadly, the goal of the research can be summed up by a famous Galileo Galilei quote as an effort to "measure what is measurable and make measurable what is not so". In this instance the overall objective was to explore the uses of chromatography and mass spectrometry to explore public and environmental health. As described in the abstract this very broad statement was refined into three mains areas concerning analyte chirality, improving the analysis of current biomarkers and developing new biomarkers of public health.

Aim one: To explore the uses of chirality in determining analyte origin and its impact on assessments of public and environmental health

Despite many pharmaceuticals containing a chiral centre, when they are examined in wastewater [1-4] or the environment [5] they are often only analysed achirally, as a sum of their constituent isomers, instead of as individual isomers. Human metabolism is often stereoselective preferentially metabolising one isomer of a parent molecule over another. This means that chirality can be used to confirm that analytes detected in wastewater or river water are being consumed by the population rather than simply being discharged into the system [6; 7]. Establishing the origins of an analyte is important when assessing both public and environmental health. For example, strategies to reduce public consumption of an environmentally toxic pharmaceutical may be misplaced if it originated from direct discharge into the environment rather than from human consumption. Chapter one concerns a five-year study of wastewater, which was carried out using chiral chromatography allowing for the confirmation that analytes present in wastewater were being consumed rather than disposed of via the sewage system. This was particularly important for monitoring MDMA, which has been previously detected in literature at high concentrations due to disposal [8]. Chirality can also be used to determine the method of manufacture of an analyte [9], which is particularly important for determining the supply route used to import drugs of abuse into the UK or Europe [10]. Where chapter one primarily focused on human health chapter two focused on the determining the environmental fate of ephedrine, a pharmaceutical with two chiral centres, by exploring the stability and toxicity of each isomer individually. River simulating microcosms containing all four ephedrine isomers were used to explore the degradation of ephedrine under light, dark, biotic and abiotic conditions and were followed up by individual isomer microcosms under the conditions that gave the greatest amount of degradation. Toxicity testing was performed by exposing *Daphnia magna*, *Pseudokirchneriella subcapita* and *Tetrahymena thermophila* to individual ephedrine isomers and determining the EC_{50}. Additionally, human liver microcosms and retrospective analysis of river water samples were used to explore the potential for non-medicinal ephedrine isomers to enter the environment as a result of human metabolism of medicinal ephedrine isomers, or from metabolism of similar compounds such as cathinones. As was the case with using chirality to determine the source of analytes in wastewater, HLM was used to help gain a better understanding of human metabolism of ephedrine as many studies of ephedrine metabolism were performed in the previous century using achiral methodologies.

Aim two: To develop new methodologies to maximise the information obtainable from currently utilised health biomarkers

A key part of assessing public health is the context in which the results should be viewed. WBE is still a relatively new field and whilst, at time of writing, key biomarkers have been surveyed for fifteen years [11] the relationship between what is detected in wastewater and public health is still not fully understood. This is particularly important as WBE has a unique capability to provide feedback on how public health and other initiatives directly impact the public, which are otherwise difficult to

measure. In particular, the UK drug policy commission (UKDPC) and advisory council on the misuse of drugs (AMCD) have highlighted the need for better methods of assessing the impact of UK drug policy [12; 13]. Likewise, many countries are currently experiencing an epidemic of opioid misuse, which is initially characterised by abuse of prescription painkillers that changes into abuse of illicit opioids like heroin [14-16]. There are also concerns surrounding the joint abuse of heroin and benzodiazepines [17]. In addition to expressing the importance of chirality, chapter one also compared trends in UK drug use with those of a larger, longer, European wide WBE study [2] to assess which trends were likely UK specific and which were shared across the continent. However, the main objective of the paper was to establish the power of comparing local prescription and wastewater data in order to identify and provide context for longer term trends in pharmaceutical consumption. This was used to compare the prescription of pharmaceuticals that are of risk of illicit abuse, such as painkillers, to their concentration in wastewater in order to identify any potential abuse that could relate to other opioid crises. This technique was also used to estimate heroin consumption by subtracting licit sources of morphine, like prescribed morphine and codeine, from wastewater concentrations of morphine in order to calculate the amount of morphine produced from heroin metabolism and hence the amount of heroin consumed. As well as being important for giving context to WBE, analysis of chirality is also important for monitoring environmental health, as many CEC enter the environment through discharge of treated wastewater [18], making it important to monitor not just environmental matrices but also wastewater. Despite this there is a lack of methods for chiral analysis of CECs. Chapter three explored the use of supercritical fluid chromatography (SFC) for the chiral analysis of CECs in wastewater and river water. SFC has several advantages over traditional liquid chromatography including more efficient analyte separation as a result of its compatibility with a wider range of stationary phases, including chiral stationary phases. Having previously highlighted the importance of chiral chromatography, chapter three detailed the steps required for developing and validate a quantitative method to analyse 95 CECs using chiral-SFC coupled to triple quadrupole mass spectrometry in influent wastewater, effluent wastewater and river water. Additional details of initial method development using a single quadrupole MS for qualitative analysis of 140 CECs was also reported in this chapter in order to highlight the range of analytes that can be analysed by SFC and to show the selectivity of different chiral SFC stationary phases to a range of chiral analytes.

Aim three: The exploration of proteins as potential new biomarkers of public health

Since its inception WBE has focussed primarily on drugs of abuse and pharmaceuticals [9; 19-21]. This has likely been because they are relatively easy to analyse using already established techniques such as reverse-phase liquid chromatography and triple quadrupole or quadrupole-time of flight mass spectrometry. However, these biomarkers are focused on measuring human consumption and relating that to public health instead of trying to measure public health directly. Where human health biomarkers have been analysed, they have been biomarkers of broad health conditions, such as

biomarkers of oxidative stress [22; 23], rather than biomarkers of specific diseases. Therefore, there exists an opportunity to explore new biomarkers that focus on truly monitoring public heath, rather than monitoring public consumption. Chapter four highlights the possibility of using urinary proteins as biomarkers of public health, which is simplified because they are already analysed clinically using similar techniques and instrumentation to other WBE biomarkers [24]. The challenge that urinary proteins pose however is that they have generally only been used previously to assess individual health, and few attempts have been made to use them for monitoring public health [25]. Additionally, in order to analyse proteins in a similar fashion to other WBE biomarkers they must first be broken down into peptides using an enzyme. Chapter five outlines the initial development of a methodology to analyse proteins in wastewater using a trypsin enzyme digest to generate peptides, followed by peptide analysis using hydrophilic interaction liquid chromatography coupled to a triple quadrupole mass spectrometer. The digest was first developed in buffer and focussed on five different protein biomarkers identified in chapter four as being potential candidates for wastewater analysis, with further development expanding the digest for use in influent wastewater. In addition, chapter five also discussed the issue of ensuring biomarker specificity in wastewater where proteins can have non-anthropogenic sources and detailed a calculation for estimating the wastewater concentration of a protein from its urinary concentration in healthy individuals, which is important when considering future biomarker candidates.

Abbreviations

Absolute quantification (AQUA)
Acetonitrile (MeCN)
Advisory Council on the Misuse of Drugs (ACMD)
Analysis of Variance (ANOVA)
Anterior Gradient protein 2 (AGR2)
Atmospheric Pressure Chemical Ionisation (APCI)
Bovine Serum Albumin (BSA)
British National Formulary (BNF)
Cardiovascular Disease (CVD)
Cellobiohydrolase (CBH)
Chemical Oxygen Demand (COD)
Chronic Kidney Disease (CKD)
Compounds of environmental concern (CEC)
Concentration factor (CF)
C-Reactive Protein (CRP)
Dark Abiotic (DAR)

Dark Biotic (DBR)
Diastereomeric Fraction (DF)
Dissolved Oxygen (DO)
Dithiothreitol (DTT)
Drug Target Residues (DTR)
Drugs of Abuse (DOA)
Electrospray Ionisation (ESI)
Enantiomeric Fraction (EF)
Environmental Protection Agency (EPA)
Environmental Risk Assessment (ERA)
Enzyme-linked immunosorbent assay (ELISA)
Ethanol (EtOH)
European Monitoring Centre for Drugs and Drug Addiction (EMCDDA)
Extracted Ion Chromatogram (EIC)
Food and Drug Administration (FDA)
Formic acid (FA)
Gas Chromatography (GC)
General Practitioner (GP)
Guanidinium chloride (GuHCl)
High Density Polyethylene (HDPE)
High Performance Liquid Chromatography (HPLC)
Human Liver Microsomes (HLMs)
Hydrophilic Interaction Liquid Chromatography (HILIC)
Interleukin-6 (IL-6)
Interleukin-8 (IL-8)
Iodoacetamide (IAM)
Iodoacetic Acid (IAA)
Isopropyl Alcohol (IPA)
Light Abiotic (LAR)
Light Biotic (LBR)
Limit of Detection (LOD)
Limit of Quantification (LOQ)
Liquid Chromatography (LC)
Liquid-Liquid Extraction (LLE)
Mass Spectrometry (MS)

Methanol (MeOH)

Molecular Weight Cut-Off (MWCO)

Multiple-Reaction Monitoring (MRM)

National Health Service (NHS)

Non-steroidal Anti-Inflammatory Drug (NSAID)

Novel Psychoactive Substances (NPS)

Optical Density (OD)

Phosphodiesterase-5 inhibitors (PDE5)

Podocin (PDM)

Points Per Peak (PPP)

Poly Vinyl Difluoride (PVDF)

Population Normalised Daily Load (PNDL)

Population Normalised Daily Prescription (PNDP)

Proof of Concept (PoC)

Prostate Specific Antigen (PSA)

Proteins Standards for Absolute Quantification (PSAQ)

Quadrupole – Time of Flight (Q-ToF)

Relative Standard Deviation (RSD)

Reverse Phase (RP)

River Water (RW)

Signal Suppression (SS)

Sodium Deoxycholate (SDC)

Sodium Dodecyl Sulphate (SDS)

Solid Phase Extraction (SPE)

Standard Deviation of the intercept (SDC)

Supercritical Fluid Chromatography (SFC)

Trifluoracetic acid (TFA)

Trifluoroethanol (TFE)

Triple quadrupole (QQQ)

Tris(2-carboxyethyl)phosphine (TCEP)

UK Drug Policy Commission (UKDPC)

Ultra (High) Performance Liquid Chromatography (U(H)PLC)

Ultra-Performance Convergence Chromatography (UPC^2)

Urban Water Fingerprinting (UWF)

Uromodulin (URM)

Wastewater (WW)

Wastewater Based Epidemiology (WBE)

Wastewater Treatment Plant (WWTP)

Water Framework Directive (WFD)

References:

[1] Baz-Lomba, J.A., Salvatore, S., Gracia-Lor, E., Bade, R., Castiglioni, S., Castrignano, E., Causanilles, A., Hernandez, F., Kasprzyk-Hordern, B., Kinyua, J., McCall, A.K., van Nuijs, A., Ort, C., Plosz, B.G., Ramin, P., Reid, M., Rousis, N.I., Ryu, Y., de Voogt, P., Bramness, J. and Thomas, K., 2016. Comparison of pharmaceutical, illicit drug, alcohol, nicotine and caffeine levels in wastewater with sale, seizure and consumption data for 8 European cities. *Bmc Public Health,* 16.
[2] Gonzalez-Marino, I., Baz-Lomba, J.A., Alygizakis, N.A., Andres-Costa, M.J., Bade, R., Barron, L.P., Been, F., Berset, J.D., Bijlsma, L., Bodik, I., Brenner, A., Brock, A.L., Burgard, D.A., Castrignano, E., Christophoridis, C.E., Covaci, A., de Voogt, P., Devault, D.A., Dias, M.J., Emke, E., Fatta-Kassinos, D., Fedorova, G., Fytianos, K., Gerber, C., Grabic, R., Gruner, S., Gunnar, T., Hapeshi, E., Heath, E., Helm, B., Hernandez, F., Kankaanpaa, A., Karolak, S., Kasprzyk-Hordern, B., Krizman-Matasic, I., Lai, F.Y., Lechowicz, W., Lopes, A., de Alda, M.L., Lopez-Garcia, E., Love, A.S.C., Mastroianni, N., McEneff, G.L., Montes, R., Munro, K., Nefau, T., Oberacher, H., O'Brien, J.W., Olafsdottir, K., Pico, Y., Plosz, B.G., Polesel, F., Postigo, C., Quintana, J.B., Ramin, P., Reid, M.J., Rice, J., Rodil, R., Senta, I., Simoes, S.M., Sremacki, M.M., Styszko, K., Terzic, S., Thomaidis, N.S., Thomas, K.V., Tscharke, B., van Nuijs, A.L.N., Yargeau, V., Zuccato, E., Castiglioni, S. and Ort, C., 2020. Spatio-temporal assessment of illicit drug use at large scale: evidence from 7 years of international wastewater monitoring. *Addiction,* 115(1), pp. 109-120.
[3] Gonzalez-Marino, I., Gracia-Lor, E., Rousis, N.I., Castrignano, E., Thomas, K.V., Quintana, J.B., Kasprzyk-Hordern, B., Zuccato, E. and Castiglioni, S., 2016. Wastewater-Based Epidemiology To Monitor Synthetic Cathinones Use in Different European Countries. *Environmental Science & Technology,* 50(18), pp. 10089-10096.
[4] Rousis, N.I., Gracia-Lor, E., Zuccato, E., Bade, R., Baz-Lomba, J.A., Castrignano, E., Causanilles, A., Covaci, A., de Voogt, P., Hernandez, F., Kasprzyk-Hordern, B., Kinyua, J., McCall, A.K., Plosz, B.G., Ramin, P., Ryu, Y., Thomas, K.V., van Nuijs, A., Yang, Z.G. and Castiglioni, S., 2017. Wastewater-based epidemiology to assess pan-European pesticide exposure. *Water Research,* 121, pp. 270-279.
[5] Petrie, B., Youdan, J., Barden, R. and Kasprzyk-Hordern, B., 2016. Multi-residue analysis of 90 emerging contaminants in liquid and solid environmental matrices by ultra-high-performance liquid chromatography tandem mass spectrometry. *Journal of Chromatography A,* 1431, pp. 64-78.
[6] Petrie, B., Youdan, J., Barden, R. and Kasprzyk-Hordern, B., 2016. New Framework To Diagnose the Direct Disposal of Prescribed Drugs in Wastewater - A Case Study of the Antidepressant Fluoxetine. *Environmental Science & Technology,* 50(7), pp. 3781-3789.
[7] Depaolini, A.R., Fattore, E., Cappelli, F., Pellegrino, R., Castiglioni, S., Zuccato, E., Fanelli, R. and Davoli, E., 2016. Source discrimination of drug residues in wastewater: The case of salbutamol. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences,* 1023, pp. 62-67.
[8] Emke, E., Evans, S., Kasprzyk-Hordern, B. and de Voogt, P., 2014. Enantiomer profiling of high loads of amphetamine and MDMA in communal sewage: A Dutch perspective. *Science of the Total Environment,* 487, pp. 666-672.
[9] Castrignano, E., Yang, Z., Bade, R., Baz-Lomba, J.A., Castiglioni, S., Causanilles, A., Covaci, A., Gracia-Lor, E., Hernandez, F., Kinyua, J., McCall, A.-K., van Nuijs, A.L.N., Ort, C., Plosz, B.G., Ramin, P., Rousis, N.I., Ryu, Y., Thomas, K.V., de Voogt, P., Zuccato, E. and Kasprzyk-Hordern, B., 2018. Enantiomeric profiling of chiral illicit drugs in a pan-European study. *Water Research,* 130, pp. 151-160.

[10] European Monitoring Centre for Drugs and Drug Addiction and Europol, 2019. *EU drug markets report 2019.* Luxembourg: Publications office of the European Union.
[11] Zuccato, E., Chiabrando, C., Castiglioni, S., Calamari, D., Bagnati, R., Schiarea, S. and Fanelli, R., 2005. Cocaine in surface waters: a new evidence-based tool to monitor community drug abuse. *Environmental health : a global access science source,* 4, pp. 14-14.
[12] Iversen, L., Gibbons, S., Eden, O.M.R. and Milton, A., 2011. Consideration of the Novel Psychoactive Substances ('legal highs'). pp. 1-70.
[13] Reuter, P. and Stevens, A., 2007. Bringing evidence and analysis together to inform UK drug policy. The UK Drug Policy Commission, pp. 1-108.
[14] Champagne-Langabeer, T., Madu, R., Giri, S., Stotts, A.L. and Langabeer, J.R., 2019. Opioid prescribing patterns and overdose deaths in Texas. *Substance Abuse*.
[15] Cicero, T.J., Ellis, M.S. and Harney, J., 2015. Shifting Patterns of Prescription Opioid and Heroin Abuse in the United States. *New England Journal of Medicine,* 373(18), pp. 1789-1790.
[16] Cicero, T.J., Ellis, M.S. and Kasper, Z.A., 2020. Polysubstance Use: A Broader Understanding of Substance Use During the Opioid Crisis. *American journal of public health,* 110(2), pp. 244-250.
[17] EMCDDA, E.M.C.f.D.a.D.A., 2018. The misuse of benzodiazepines among high-risk opioid users in Eruope. pp. 1-10.
[18] Kasprzyk-Hordern, B. and Baker, D.R., 2012. Enantiomeric Profiling of Chiral Drugs in Wastewater and Receiving Waters. *Environmental Science & Technology,* 46(3), pp. 1681-1691.
[19] Kim, K.Y., Lai, F.Y., Kim, H.Y., Thai, P.K., Mueller, J.F. and Oh, J.E., 2015. The first application of wastewater-based drug epidemiology in five South Korean cities. *Science of the Total Environment,* 524, pp. 440-446.
[20] Lai, F.Y., O'Brien, J., Bruno, R., Hall, W., Prichard, J., Kirkbride, P., Gartner, C., Thai, P., Carter, S., Lloyd, B., Burns, L. and Mueller, J., 2016. Spatial variations in the consumption of illicit stimulant drugs across Australia: A nationwide application of wastewater-based epidemiology. *Science of the Total Environment,* 568, pp. 810-818.
[21] Gao, T.T., Du, P., Xu, Z.Q. and Li, X.Q., 2017. Occurrence of new psychoactive substances in wastewater of major Chinese cities. *Science of the Total Environment,* 575, pp. 963-969.
[22] Ryu, Y., Gracia-Lor, E., Bade, R., Baz-Lomba, J.A., Bramness, J.G., Castiglioni, S., Castrignano, E., Causanilles, A., Covaci, A., de Voogt, P., Hernandez, F., Kasprzyk-Hordern, B., Kinyua, J., McCall, A.K., Ort, C., Plosz, B.G., Ramin, P., Rousis, N.I., Reid, M.J. and Thomas, K.V., 2016. Increased levels of the oxidative stress biomarker 8-iso-prostaglandin F-2 alpha in wastewater associated with tobacco use. *Scientific Reports,* 6.
[23] Sims, N., Rice, J. and Kasprzyk-Hordern, B., 2019. An ultra-high-performance liquid chromatography tandem mass spectrometry method for oxidative stress biomarker analysis in wastewater. *Analytical and Bioanalytical Chemistry,* 411(11), pp. 2261-2271.
[24] Parker, C.E. and Borchers, C.H., 2014. Mass spectrometry based biomarker discovery, verification, and validation - Quality assurance and control of protein biomarker assays. *Molecular Oncology,* 8(4), pp. 840-858.
[25] Stuveling, E.M., Hillege, H.L., Bakker, S.J.L., Gans, R.O.B., de Jong, P.E. and de Zeeuw, D., 2003. C-reactive protein is associated with renal function abnormalities in a non-diabetic population. *Kidney International,* 63(2), pp. 654-661.

This declaration concerns the article entitled:	
Wastewater-based epidemiology combined with local prescription analysis as a tool for temporal monitoring of drugs trends - a UK perspective	
Publication status (tick one) **Draft manuscript** [ ] **Submitted** [ ] **In review** [ ] **Accepted** [ ] **Published** [x]	
Publication details (reference)	DOI: 10.1016/j.scitotenv.2020.139433 Rice,. J., Kannan, AM., Castrignanò, E., Jagadeesan, K., Kasprzyk-Hordern, B., 2020. Wastewater-based epidemiology combined with local prescription analysis as a tool for temporal monitoring of drug trends – A UK perspective. *Science of the Total Environment*, 735
Copyright status (tick the appropriate statement) I hold the copyright for this material [ ] Copyright is retained by the publisher, but I have been given permission to replicate the material here [x]	
Candidate's contribution to the paper (provide details, and also indicate as a percentage)	Formulation of ideas: Candidate considerably contributed to the formulation of ideas concerning the collation and interpretation of data, in collaboration with Barbara Kasprzyk-Hordern (BKH) Kishore Jagadeesan (KJ) considerably contributed to the formulation of ideas concerning prescription data with BKH Design of methodology: Erika Castrignanò (ECA) predominantly executed the development and validation of the analytical method used in the paper KJ predominantly executed the design of the PDA tool for collation of prescription data The candidate predominantly executed the design of how data from 2014-2018 should be collated and interpreted and how data from the SCORE network should be used to interpret this data The candidate significantly contributed to how prescription data would be interpreted alongside collated data Experimental work: ECA predominantly executed the collected, analysis and interpreted data for 2014-2015 for her own research, and did not interpret data for this paper The candidate predominantly executed the collection, analysis and interpretation of data for 2016-2017 and the interpretation of data for 2018. The candidate then collated all data from 2014-2018 and interpreted the data as a whole and as used for this paper

	Andrew Kannan (AK) predominantly executed the collection and analysis of data for 2018 The SCORE network predominantly executed to analysis and collation of related European monitoring data from 2011-2018 to which ECA contributed from 2014-2015, the candiatie contributed from 2016-2017 and AK contributed in 2018 KJ predominantly executed the development and implementation of the PDA tool designed to gather prescription data. KJ predominantly executed the collation of data on monthly prescription rates for pharmaceuticals specified by the candidate The candidate predominantly executed the interpretation of pharmaceutical data with regards to the collated 2014-2018 data. Presentation of data in journal format: Candidate predominantly executed the writing and formatting of all data for publication, in collaboration with BKH, ECA, KJ and AK KJ contributed to the writing of prescription collection methodologies
Statement from Candidate	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature.
Signed	 **Date** 02/06/2020

Chapter one introduction

In the thesis introduction the three aims of the research were laid out and can be broadly summarised as follows: 1) to demonstrate the importance of analyte chirality, 2) to acquire better data using current health biomarkers and 3) to explore proteins as potential new biomarkers of human health. To this end, chapter one explores both the importance of measuring chirality for identifying the source of chiral analytes and the novel use of local prescription data to provide context to WBE data by reporting the results of a five year study of influent wastewater, which was undertaken from 2014-2018, with wastewater being collected once a day for one week each year.

The paper reports on the results of a study of wastewater performed for one week each march from 2014-2018 and analysed using a chiral chromatography method. The paper aims to identify trends in the consumption of drugs of abuse and pharmaceuticals and uses a combination of local prescription data, European wastewater data and changes in enantiomeric fraction (EF) in order to give context to the results.

The importance of using chiral chromatography for collecting chiral information will be demonstrated in how it allows for the confirmation that detected analytes were being consumed. It achieves this by a comparison between the EF of analytes in wastewater and the EF of analytes in urine, as reported in literature. Likewise, local prescription data represents an important source of data with regards to the supply for pharmaceuticals and would be expected to match with trends in wastewater concentration if a substance is being us as intended. This is particularly important for prescription pharmaceuticals at risk of being abused, which could be potentially identified by an increase in wastewater concentrations relative to the prescribed concentration. For illicit substances context is provided by comparison with the larger European study, where possible, in order to identify UK specific and UK non-specific trends in drug use. This will demonstrate the power of WBE in providing data on the efficacy of public health initiatives, such as the regulation of illicit substances, by measuring what is being consumed by the population and how that can change over time.

Wastewater-based epidemiology combined with local prescription analysis as a tool for temporal monitoring of drugs trends - a UK perspective

Jack Rice[a], *Andrew M. Kannan*[a], *Erika Castrignanò*[a,b], *Kishore Jagadeesan*[a] *and Barbara Kasprzyk-Hordern*[a*]

[a] Department of Chemistry, Faculty of Science, University of Bath, Bath, BA2 7AY, United Kingdom (UK)

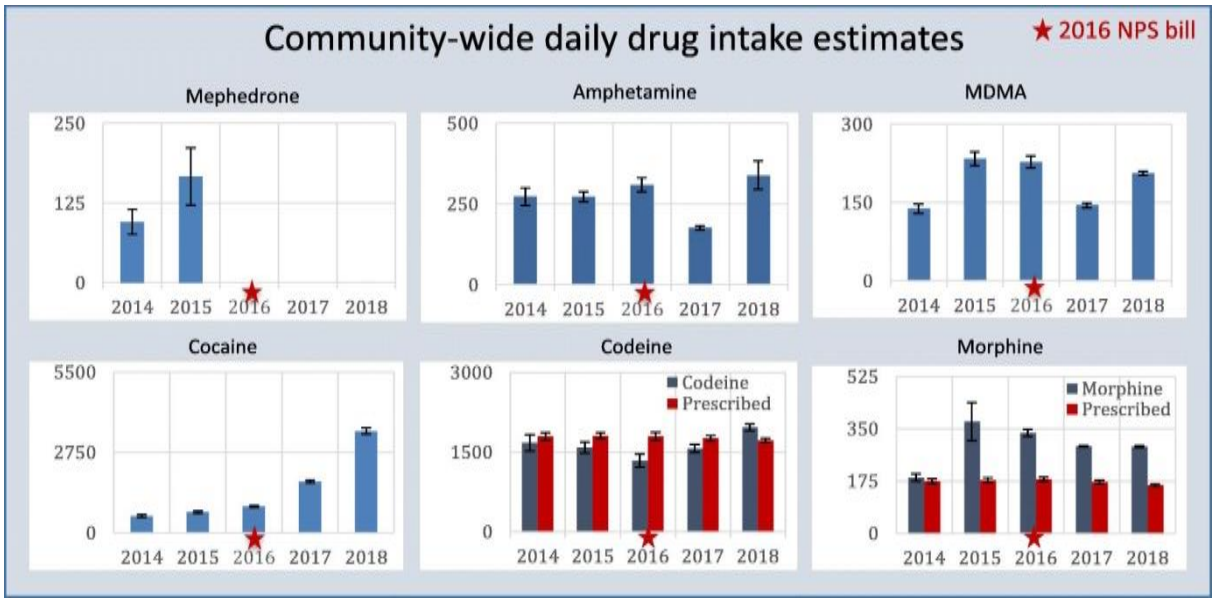
[b] Current address: Department of Analytical, Environmental & Forensic Sciences, School of Population Health & Environmental Sciences, King's College London, London SE1 9NH

***** Corresponding author: Barbara Kasprzyk-Hordern, E-mail: b.kasprzyk-hordern@bath.ac.uk

Abstract:

This paper reports the application of wastewater-based epidemiology (WBE) for the monitoring of one city in the UK in years 2014-2018 as a means of 1) exploring relative temporal changes of illicit drug usage trends across 5 sampling weeks in 5 years, (2) assess policy impact in reducing drug consumption, focussing particularly on mephedrone, which was classified as a class B drug in the UK in 2010, and the effects of subsequent regulation such as the novel psychoactive substances (NPS) bill of 2016, (3) investigate temporal changes in consumption of prescription pharmaceuticals vs illicit drug usage, and (4) compare consumption of prescription drugs with WBE to enable more accurate verification of prescription drugs with abuse potential. Mephedrone was quantified only for the first two years of the study, 2014-2015, and remained undetected for the next three years of the study. This shows that given enough time changes in drug policy can have an effect on drug consumption. However, after the introduction of the 2016 NPS bill, between the third and fourth study years, there was an observable increase in the consumption of "classic" drugs of abuse such as cocaine, MDMA and ketamine suggesting a shift away from novel psychoactives. The unique prescription dataset allowed for a more accurate calculation of heroin consumption using morphine by examining other sources morphine. Additionally, for compounds with controlled prescription like methadone, trends in consumption estimated by wastewater and trends in prescription correlated. Wastewater-based epidemiology is a powerful tool for examining whole populations and determining the efficacy and direction of government actions on health, as it can, alongside prescription and wider monitoring data, provide a clear insight into what is being consumed by a population and what action is needed to meet required goals.

Key words: wastewater-based epidemiology, illicit drugs, mephedrone, opioids



1. Introduction

In 2017 the UK home office published its drug strategy and set out its aims of reducing illicit and other harmful drug use and increasing rates of recovery from drug dependence (Home Office 2017). This was to be achieved by focussing on reducing demand for, restricting supply of, building recovery from and global actions on drugs of abuse. However the efficacy of policies designed to achieve this are difficult to measure by traditional means, such as surveys, police seizures and hospital records. This was discussed in reports from the UK drug policy commission (UKDPC) (Reuter and Stevens 2007) and the advisory council on the misuse of drugs (ACMD) (Iversen, Gibbons et al. 2011). The UKDPC highlights concerns regarding underreporting the number of drug users, and hence the scope of drug use, is a problem with current survey-based methods of data collection. The ACMD further highlights the difficulty in obtaining an accurate picture on drug use, particularly for novel psychoactive substances, such as mephedrone, where users may not be aware what they are taking. The UK 2017 drug strategy also aims to engage problematic drug users to encourage abstinence as part of reducing demand for, and increasing recovery from drugs of abuse. However, the UKDPC highlights that vulnerable drug users would not engage with social services, meaning that perhaps the most important group of drug users would not be captured by hospital or survey data. Lastly, whilst police seizures or festival amnesty bins can allow for an understanding of drug purity, drug supply and drug availability they cannot necessarily provide a complete picture of the drug market without seizing all the drugs on the market (Reuter and Stevens 2007).

Traditional data collection approaches rely on obtaining accurate information from incomplete data sources about individual behaviour and then extrapolating them to understand the whole community, which can be misleading. Instead of this bottom-up approach of understanding the community by understanding some of its members, wastewater-based epidemiology (WBE) allows for a top-down approach by examining the whole community using community-derived wastewater (Ort, van Nuijs et al. 2014, Baz-Lomba, Salvatore et al. 2016, Castrignano, Yang et al. 2018, Gonzalez-Marino, Baz-Lomba et al. 2020). The advantage of WBE is that information can be rapidly collected and analysed to provide near real time monitoring of a community. As all individuals, whether they consume illicit drugs or not, contribute to the wastewater it allows for an understanding of the drugs used within the community from a single, representative, anonymous sample. This can be used to understand short- and long-term trends in drug use, and in combination with an understanding of drug metabolism and police seizure data, this would allow for an understanding of the whole drug market. In effect, WBE could provide information on what is consumed, whilst police seizures on what is not. A disadvantage of WBE is that it cannot provide information on drug purity or the number of drug users, without additional information such as dosage, frequency of use and metabolomics excretion data. However if the goal of policy is to reduce the number of users then that can be determined by a corresponding decrease in the amount of consumption using WBE. Additionally, WBE can be used to examine the consumption of pharmaceuticals and other licit drugs, which combined with additional data sources, like prescription data from the National Health Service (NHS). Prescriptions can also provide a valuable insight into the use of pharmaceuticals that have the potential to be abused, such as opioids. The European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) UK drug report highlights that the number of heroin users is decreasing, but there are increasing concerns about abuse of prescription opioids and the numbers entering specialised treatment for heroin addiction have been static since 2014 (Addiction 2018). The use of additional non-wastewater data is necessary to help validate long term trends in WBE, particularly studies when trying to establish temporal trends across years from only a single week (Ort, Eppler et al. 2014, Goulding, Hickman et al. 2020).

In January 2016 the psychoactive substances (NPS) bill was given royal assent and entered UK law in May 2016 (Home Office 2015). The NPS bill covered all psychoactive substances for human consumption that were not already controlled by the 1971 misuse of drugs act, or

otherwise exempted. This served to effectively ban all products by exception and supported the government's stated aims of reducing the supply of illicit drugs by preventing the previously legal grey area surrounding the sale of psychoactive substances in head shops (Office 2015). In 2018, the government carried out a review of the 2016 NPS act to assess its impacts on curbing the use of NPS (Office 2018). The report largely reasons that the act was a success, leading to a decrease in the availability of NPS through the restriction of supply and a cessation of the "open" selling of NPSs. However, the report highlights that a lot of this evidence is qualitative, as the supply of NPSs has shifted from head shops to more illicit sources including street dealing and the dark web. Additionally, whilst there has been a decrease in NPS usage, particularly amongst young people (16-24), there has been an increase in the usage of Class A drugs from 2016-2018, and new NPSs continue to be developed and appearing in the UK. The report suggests that due to the global nature of the illicit drug market it is difficult to say if this is due to the NPS act or to wider trends in the drug market.

Therefore, there exists an opportunity to use WBE to better understand drug use in the UK. From 2014 to 2018 a large city in the South-West of the UK was surveyed for one week each year in order to contribute data on illicit drug use to a European project (EMCDDA 2019, Gonzalez-Marino, Baz-Lomba et al. 2020). Whilst this study was only interested in four analytes data was collected on a much greater number of analytes over a period that, fortuitously, included a major piece of legislation that sought to fundamentally change how licit and illicit substances were regulated – the 2016 NPS act. This paper seeks to understand the use of licit and illicit substances and how this may have changed in the light of significant legislation.

This paper aims to estimate illicit and legal drug usage trends in one city in the UK from 2014 to 2018 via application of wastewater-based epidemiology and pharmaceutical prescription data for the very same city and study time to: (1) explore relative temporal changes of illicit drug usage trends across 5 sampling weeks in 5 years, (2) assess policy impact in reducing drug consumption, focussing particularly on mephedrone, which was classified as a class B drug in the UK in 2010, and the effects of subsequent regulation such as the novel psychoactive substances (NPS) bill of 2016, (3) investigate temporal changes in consumption of prescription pharmaceuticals vs illicit drug usage, and (4) compare consumption of prescription drugs with WBE to enable more accurate verification of prescription drugs with abuse potential.

2. Experimental

2.1.Materials

Over the five years of the study the following analytes were selected (Table 1): opioid analgesics, benzodiazepines, antidepressants, stimulants, hallucinogens, dissociative, designer drugs, PDE5 inhibitors, Z-drugs, lifestyle biomarkers. Table S1 shows all target analytes, their CAS number, molecular formula, molecular weight, and supplier information.

Table 1. Analytes selected in this study.

Analyte	Class of compound	Internal standard
1,7-dimethylxanthine (1,7-DMX)	Lifestyle biomarker/Metabolite (of caffeine)	Cotinine-d3
7-Aminonitrazepam (AMNITRA)	Benzodiazepine/Metabolite (of nitrazepam)	Nitrazepam-d5
Amitriptyline (AMI)	Antidepressant	EDDP-d3
Amphetamine (AMP)	Stimulant	Amphetamine-d5
Anhydroecgonine methyl ester (AEME)	Stimulant/Metabolite (of cocaine)	Cocaine-d3
Benzoylecgonine (BEG)	Stimulant/Metabolite (of cocaine)	Benzoylecgonine-d8
Benzylpiperazine (BZP)	Stimulant	Phencyclidine-d5
Caffeine (CAFE)	Lifestyle biomarker	Cotinine-d3
Cocaethylene (CE)	Stimulant/Metabolite (of cocaine & alcohol co-consumption)	Cocaethylene-d3
Cocaine (COC)	Stimulant	Cocaine-d3
Codeine (COD)	Opioid	Codeine-d6
Cotinine (COT)	Lifestyle biomarker/Metabolite (of nicotine)	Cotinine-d3
Creatinine (CREAT)	Lifestyle biomarker	Cotinine-d3
Desmethylvenlafaxine (DMVENLA)	Antidepressant/Metabolite (of venlafaxine)	Methamphetamine-d5
2-(3,4-Dihydroxyphenyl)-N-methylpropylamine (DHMA)	Hallucinogen/Metabolite (of MDMA)	Amphetamine-d5
Desmethylvenlafaxine	Antidepressant/metabolite (of Venlafaxine)	Methamphetamine-d5
Diazepam (DZP)	Benzodiazepines	Diazepam-d5
Dihydrocodeine (DHCOD)	Opioid/Metabolite (of codeine)	Codeine-d6
Dihydromorphine (DHMORPH)	Opioid/Metabolite(of morphine)	Morphine-d6
2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP)	Opioid/Metabolite (of methadone)	EDDP-d3
Ephedrine (EPH)	Stimulant/Drug precursor	1S,2R-(+)-ephedrine-d3
Fluoxetine (FLOX)	Antidepressant	Fluoxetine-d5
Heroin (HEROIN)	Opioid	Heroin-d9
1,4-Hydroxy-3-methoxyampheamine (HMA)	Hallucinogen/Metabolite (of MDMA)	Amphetamine-d5
1,4-Hydroxy-3-methoxymethamphetamine (HMMA)	Hallucinogen/Metabolite (of MDMA)	Methamphetamine-d5
Hydrocodone (HYCDNE)	Opioid	Hydrocodone-d6
Ketamine (KETA)	Dissociative agent	Ketamine-d4
Lorazepam (LORAZE)	Benzodiazepine	Lorazepam-d4
3,4-Methylene dioxyethyl amphetamine (MDA)	Hallucinogen/Metabolite (of MDMA)	MDA-d5
3,4-Methylene dioxyethyl methamphetamine (MDMA)	Hallucinogen	MDMA-d5

3,4-Methylene dioxyethyl ethylamphetamine (MDEA)	Hallucinogen	MDEA-D5
Mephedrone (DRONE)	Stimulant	Mephedrone-d3
Methadone (MDONE)	Opioid	Methadone-d9
Methamphetamine (METH)	Stimulant	Methamphetamine-d5
Morphine (MORPH)	Opioid	Morphine-d6
Morphine-3b-D-glucuronide	Opioid/metabolite (of Morphine)	Morphine-3b-D-glucuronide-d3
Nicotine (NIC)	Lifestyle biomarker	Cotinine-d3
Nitrazepam (NITRA)	Benzodiazepine	Nitrazepam-d5
Norcodeine (NorCOD)	Opioid/Metabolite (of codeine)	Codeine-d6
N-desmethyl-cis-tramadol (NDMTRAM)	Opioid/metabolite (of tramadol)	Codeine-d6
Nordiazepam (NorDZP)	Benzodiazepines/Metabolite (of diazepam)	Nordiazepam-d5
Norephedrine (NorEPH)	Stimulant/Metabolite (of ephedrine)	1S,2R-(+)-ephedrine-d3
Norfluoxetine (NorFLOX)	Antidepressant/Metabolite (of fluoxetine)	Fluoxetine-d5
Norketamine (NorKETA)	Dissociative agent/Metabolite (of ketamine)	Norketamine-d4
Normorphine (NorMORPH)	Opioid/Metabolite (of morphine)	Morphine-d6
Noroxycodone (NorOXY)	Opioid/Metabolite (of oxycodone)	Oxycodone-d6
Nortriptyline (NTRIP)	Antidepressant	EDDP-d3
6-monoacetylmorphine (O-6-MAM)	Opioid/Metabolite (of heroin)	Codeine-d6
O-desmethyltramadol (O-DMTRAM)	Opioid/Metabolite (of tramadol)	Codeine-d6
Oxazepam (OXAZE)	Benzodiazepine	Oxazepam-d4
Oxycodone (OXY)	Opioid	Oxycodone-d6
Oxymorphone (OMP)	Opioid	Oxycodone-d6
4-methoxyamphetamine (PMA)	Psychedelic	MDA-d5
Pseudoephedrine (PSE)	Decongestant/Stimulant/Precursor	1S,2R-(+)-ephedrine-d3
Sildenafil (SILD)	Phosphodiesterase Type 5 inhibitor	EDDP-d3
Temazepam (TEMAZE)	Benzodiazepines	Temazepam-d5
Tramadol (TRAM)	Opioid	Methamphetamine-d5
Vardenafil (VARDEN)	Phosphodiesterase Type 5 inhibitor	Methadone-d9
Venlafaxine (VENLA)	Antidepressant	Methamphetamine-d5
Zolpidem (ZOLP)	Z-drugs	E1-Zopiclone-d4
Zopiclone (ZOPI)	Z-drugs	Zopiclone-d4

Deuterated analogues of target analytes were used as internal standards (IS) as detailed in Table 1: amphetamine-d5, benzoylecgonine-d8, cocaine-d3, cocaethylene-d3, codeine-d6, cotinine-d3, diazepam-d5, ecgonine methyl ester-d3, EDDP-d3, 1S,2R-(+)-ephedrine-d3, heroin-d9, hydrocodone-d6, ketamine-d4, lorazepam-d4, MDA-d5, MDEA-d5, MDMA-d5, mephedrone-d3, methadone-d9, methamphetamine-d5, morphine-d6, morphine-3-D-

glucuronide-d3, nordiazepam-d5, norketamine-d4, oxazepam-d4, oxycodone-d6, temazepam-d5 and zopiclone-d4.

The following analytes were purchased and used as racemates: (±)-mephedrone, (±)-4-hydroxy-3-methoxymethamphetamine (HMMA), (±)-3,4-methylenedioxymethamphetamine (MDMA), (±)-4-hydroxy-3-methoxyamphetamine (HMA), (±)-methamphetamine, (±)-amphetamine, (±)-3,4-methylenedioxyamphetamine (MDA), (±)-tramadol, (±)-desmethylvenlafaxine, (±)-venlafaxine, (±)-3,4-methylenedioxy-N-ethyl-amphetamine (MDEA), (±)-ephedrine, (±)-pseudoephedrine, (±)-para-methoxyamphetamine (PMA), (±)-norephedrine, (±)-norfluoxetine, (±)-zopiclone, (±)-fluoxetine, (±)-3,4-dihydroxy methamphetamine (DHMA), (±)-methadone, (±)-ketamine, (±)-norketamine, (±)-2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), (±)-lorazepam, (±)-temazepam, (±)-oxazepam.

Enantiomerically pure standard solutions were used for the following analytes: 6-monoacetylmorphine with five defined stereocentres, oxycodone ((−)-oxycodone), morphine-3-d-glucuronide, hydrocodone, dihydromorphine, codeine ((−)-codeine), morphine (d-(−)-morphine), normorphine, heroin, dihydrocodeine ((−)-dihydrocodeine), noroxycodone, oxymorphone ((−)-oxymorphone), cocaethylene, cocaine ((−)-cocaine), benzoylecgonine ((−)-benzoylecgonine) and anhydroecgonine methyl ester (AEME). Compounds synthesised, prescribed or used as racemates were purchased as racemates, whilst those prescribed or used as a single enantiomer were purchased as a single isomer. All standards and internal standards were of the highest purity available (>97%). Stock and working solutions of standards were stored at −20°C. Methanol, acetonitrile and ammonium acetate were purchased from Sigma–Aldrich, UK. Ultrapure water was obtained from MilliQ (EMD Millipore, UK). In order to prevent the adsorption of polar compounds to the hydroxyl sites on the glass surface it was treated with DMDCS to deactivate the glass. The process consisted of the following steps: rinsing of the glassware with 5% DMDCS once, with toluene twice and with methanol thrice with glassware left to dry between each rinse.

These compounds were originally selected as they represented a mixture of classical drugs of abuse, new emerging drugs of abuse and substances with abuse potential (Castrignano, Lubben et al. 2016).

2.2.Sample collection, preparation and analysis

24h composite, time proportional, wastewater influent samples were collected every day for one week each March from 2014-2018 in accordance with the procedure detailed in the following paper (Castrignano, Lubben et al. 2016). The WWTP sampled serves a large city in the South West of England equivalent to a population of 886,650 (UK city 1). The week each year selected for sampling was chosen so that it contained no significant events such as festivals, demonstrations, etc. in UK City 1, cultural events such as Saint Patrick's Day or extraordinary weather events in the South-West of the UK in line with other WBE studies (Castiglioni, Bijlsma et al. 2016). This was carried out to ensure that as close to a normal week as possible was sampled each year as public holidays will affect rates of consumption (van Nuijs, Mougel et al. 2011). Sampling always began on a Wednesday and lasted for seven days, i.e. until the following Tuesday. Wastewater flows used to calculate daily drug loads are detailed in Table S2. In brief triplicate samples of either 100 mL (2014) or 50 mL (2015-2018) aliquots of composite influent were taken and spiked with 50 µL of deuterated internal standard solution at a concentration of 1µg mL^{-1}. The pH of wastewater was not corrected as this can negatively affect the stability of certain analytes (Castiglioni and Gracia Lor 2016) and needed to be left uncorrected for inclusion in a European wide survey of wastewater (Gonzalez-Marino et al., 2020), discussed in section 3.3. The spiked samples were then filtered through Whatman GF/F filters and loaded onto conditioned Oasis HLB SPE cartridges at a rate of 3 mL min^{-1}. Cartridges were conditioned with 2 mL methanol followed by 2 mL water. After loading samples, the cartridges washed with 3 mL of MilliQ water and then left to dry under vacuum for one hour. Cartridges were then wrapped in aluminium foil and stored at -20 ^{o}C, awaiting analysis, and typically samples were stored in this way for a period of up to one month. All samples for one week were defrosted together and eluted from cartridge with 4 mL of methanol. . After elution samples were evaporated to dryness using a Turbovap evaporator under a stream of N_2 and then reconstituted in 0.5 mL of 85:15 % v/v 1 mM $NH_4OAc_{(aq)}$:MeOH and filtered through 0.2 µM PTFE syringe filters directly into the vial for analysis.

Samples were then analysed via chiral LC-MS/MS in positive electrospray ionisation (ESI+) using a ChiralPak CBH column coupled directly to a Waters Xevo TQD mass spectrometer using conditions described elsewhere (Castrignano, Lubben et al. 2016). To summarise chromatographic conditions were as follows: Isocratic mobile phase of 85:15 %v/v 1 mM $NH_4OAc_{(aq)}$:MeOH at a flow rate of 0.1 mL min^{-1}, a 100 mm x 1.0 mm, 1.7 µm ChiralPak

CBH column at 25.0 ^{o}C with a 0.2 µm PTFE pre-column filter attached. MS analysis was performed in multiple reaction monitoring mode. All samples for one year were analysed together in a single analytical run within two months of initial sample collection.

2.3. WBE back-calculations

Back calculations were performed in accordance with the procedure detailed elsewhere (Baker, Barron et al. 2014, Castrignano, Lubben et al. 2016). Briefly concentration values obtained from LC-MS analysis were used to calculate the daily load of analyte (eq. 1), the population normalised daily load (PNDL, eq. 2) and the daily intake (eq. 3). Where possible daily intake was used to compare results and where not possible PNDLs were used instead. The final two terms in equation 3, used to calculated daily intake from PNDL, are known as correction factors. Excretion and correction factors used are summarised in the table below (Table 2).

Table 2. Correction factors used for calculating daily consumption

Compound	**Drug target residue**	**Excretion (%)**	**Correction factor**	**Source**
Cocaine	Benzoylecgonine	29	3.6	(Gonzalez-Marino, Baz-Lomba et al. 2020)
Amphetamine	Amphetamine	36	2.8	
Methamphetamine	Methamphetamine	41	2.4	
MDMA	MDMA	22.5	4.4	
Mephedrone	Mephedrone	15.4	6.5	(Castrignano, Yang et al. 2018)
Pseudoephedrine	Pseudoephedrine	88.0	1.1	
Ephedrine	Ephedrine	76.9	1.3	
Heroin	O-6-MAM	1.3	86.9	(Postigo, Lopez de Alda et al. 2011)
	Morphine	42	3.08	(Gracia-Lor, Zuccato et al. 2016)
Morphine	Morphine	77.7	1.29	(Khan and Nicell 2011)
Codeine	Codeine	29.0	3.45	(Thai, O'Brien et al. 2019)
Methadone	Methadone	22.0	4.55	
Ketamine	Ketamine	30.0	3.3	(Yargeau, Taylor et al. 2014)
Tramadol	Tramadol	30.0	3.3	(Mackul'ak, Birosova et al. 2015)
Temazepam	Temazepam	74.5	1.34	(Baker, Barron et al. 2014)
Venlafaxine	Venlafaxine	5.0	20	
Dihydrocodeine	Dihydrocodeine	54.0	1.85	
Caffeine	1,7-dimethylxanthine	4.6	23.42	(Gracia-Lor, Rousis et al. 2017)
Nicotine	Cotinine	32.0	3.125	(Mackie, Tscharke et al. 2019)

Drug target residue (DTR) refers to the molecule (either drug itself or its metabolite) used to calculate daily intake (consumption) and was generally the parent molecule, except where specified, e.g. amphetamine was the DTR used for calculating daily intake of amphetamine, whilst benzoylecgonine was used instead of cocaine to calculate daily intake of cocaine (see table 2 for details). The parent molecule was always used to calculate daily load and PNDL.

$$Daily\ load\ (\mu g\ day^{-1}) = Concentration\ (\mu g\ L^{-1}) * Wastewater\ volume\ (L\ Day^{-1}) -\ eq.1$$

$$Population\ normalised\ daily\ load\ (mg\ day^{-1}1000\ inh^{-1}) = Daily\ load * 10^{-3} * \left(\frac{1000}{population}\right) -\ eq.2$$

$$Daily\ intake\ (consumption)(mg\ day^{-1}1000\ inh^{-1}) = PNDL\ * \frac{1}{Excretion} * \frac{MWparent}{MW_{DTR}} - eq.3$$

The use of non-parent molecules as DTRs was necessary for some analytes in order to accurately assess the concentration of the parent molecule. The best example of this is the use of benzoylecgonine as the DTR of cocaine. Benzoylecgonine as it is the primary metabolite of cocaine, which is heavily metabolised before excretion (Zuccato, Chiabrando et al. 2005)

Similarly, O-6-MAM is often used as the DTR of heroin (Zuccato, Chiabrando et al. 2008, Terzic, Senta et al. 2010). The metabolism of heroin and opioids in general is complex as many therapeutically used opioids are metabolites of each other (Smith 2009, Barakat, Atayee et al. 2012). Despite only a low percentage (<1.5%) of heroin being metabolised and excreted as O-6-MAM, it is used because it is a heroin specific metabolite (Postigo, Lopez de Alda et al. 2011, van Nuijs, Covaci et al. 2015). Alternatively morphine can be used (Du, Zhou et al. 2017), but requires additional information to discriminate between sources of morphine. The most comprehensive correction factor has variables include wastewater loads of morphine and codeine, the concentration and excretion rates of therapeutic codeine, morphine and the percentage of codeine and acetyl codeine in street heroin. A modified form of the back-calculation procedure used by Du et al. is used here. The basic principle of both approaches is to calculate heroin consumption from morphine by subtracting all other sources of morphine from wastewater loads of morphine. Equation 4 (eq. 4) calculates heroin consumption (mg day^{-1} 1000 inh^{-1}) from the amount of morphine attributed to heroin metabolism.

$$Heroin\ consumption = \ MOR_{HER} * \frac{1}{excretion_{MOR-HER}} * \frac{MW_{Heroin}}{MW_{Morphine}}\ eq.4$$

Where MOR_{HER} is the amount of morphine produced from heroin metabolism, which is calculated using equation 5 (eq. 5). $Excretion_{MOR\text{-}HER}$ is the excretion rate of heroin as morphine (42 %) (Gracia-Lor, Zuccato et al. 2016) and when multiplied by the ratio of heroin and morphine's molecular weights is equivalent to the correction factor (Correction factor = 3.08). MOR_{HER} can be calculated using equation 5 (eq. 5) where licit morphine (i.e. morphine prescriptions) and morphine from codeine metabolism are subtracted from the average daily intake of morphine.

$$MOR_{HER} = MOR_{WW} - MOR_{PRES} - (COD_{WW} * Excretion_{MOR-COD}) \; eq. 5$$

MOR_{WW} is the average daily intake of morphine, MOR_{PRES} is the average daily prescription rate of morphine, COD_{ww} is the average daily intake of codeine and $Excretion_{MOR\text{-}COD}$ is the amount of morphine produced from codeine metabolism (6.51%) (Khan and Nicell 2011) . The original back-calculation procedure by Du et al. examined the contribution from therapeutic and illicit sources of codeine and used PNDL of codeine and morphine instead of the daily intake. Daily intake of codeine was used under the assumption that it would contain all codeine consumed from both licit and illicit sources. However, morphine prescription may not be able to adequately capture all licit prescription of morphine due to the data being limited to prescriptions from the study city. Therefore, it was possible that users consuming licit morphine, or any other prescribed pharmaceutical, that commute into the study city would contribute licit morphine into the wastewater supply that would then be misidentified as illicit morphine, resulting in an overestimation of heroin consumption. However, as morphine is a strong painkiller for treating severe or chronic pain the number of individuals contributing licit morphine from outside the catchment is expected to be low and within other sources of method error.

Where MOR_{HER} was calculated as a negative value heroin daily intake was assumed to be <LOQ.

Data on daily wastewater volumes and the population of our study city was obtained from the city's wastewater service provider (Table S2).

2.4.Enantiomeric Fractions

Enantiomeric fractions (EFs) were calculated with respect to either the (+)-enantiomer or the first eluting enantiomer, E1, where the isomer elution order is not known. For the compounds amphetamine, methamphetamine, MDMA, MDA, ephedrine and pseudoephedrine the elution order of the isomers is known and EF was calculated using the following equation (eq. 4)

$$EF = \frac{(+) - isomer\ concentration}{(+) - isomer\ concentration + (-) - isomer\ concentration} - eq.4$$

For all other chiral molecules an alternative equation (eq. 5) was used instead

$$EF = \frac{E1 - isomer\ concentration}{E1 - isomer\ concentration + E2 - isomer\ concentration} - eq.5$$

2.5.Statistical analysis

Data is presented in the form of daily or yearly averages, which was calculated from the average from the daily averages for seven consecutive days. When comparing two averages a 2-tailed t-test was performed and significance determined at a threshold of $P \leq 0.05$ and greater significance at $P \leq 0.01$. When comparing more than two averages ANOVA was performed, again at $P \leq 0.05$ and $P \leq 0.01$ significance thresholds. Most commonly ANOVA was used to examine if there was significant variation within a week or across multiple study years before t-tests were performed. T-tests were performed on sequential years only, i.e. 2014-2015 or 2015-2016, to look for significance. T-tests on non-sequential years, i.e. 2014-2016, were only performed if ANOVA analysis showed significant variation, but sequential t-tests showed no significant variation between sequential years. Statistical analysis was performed using IBM SPSS.

Where analyte concentrations were below limits of quantification (<LOQ) a value of 0.00 was used for the purposes of ANOVA and non-sequential t-tests were used to compare years where the analyte was detected at a quantifiable concentration. Where analytes were not detected (<LOD) they were removed from ANOVA calculations.

A common phenomenon in illicit drug consumption is the presence of a so called “weekend effect”, where consumption is significantly greater on the weekend than during the week. For the purposes of calculating this Friday-Monday is considered the weekend, whilst Tuesday-Thursday is considered the weekday (EMCDDA 2020).

2.6. Prescription analysis

Mass of each compound prescribed in our study region from January 2014–November 2018 was calculated using an interactive online tool, PrAna (Jagadeesan and Kasprzyk-Hordern, in preparation), which displays all primary care prescription data in England down to the individual practice level. The data source for PrAna is the monthly prescribing datasets published by NHS Digital (https://digital.nhs.uk/). These monthly datasets provide the number of items prescribed, the net ingredient cost, actual cost and quantity by 15-digit BNF code for each practice. The tool uses quantity, which represents the quantity of a drug dispensed and mass of each compound prescribed for each practice is calculated and displayed using open source statistical software R (Team R. C., 2013). Data was provided by month and further divided by GP surgery, although this higher level of data was not required for this study and analysed using the statistical methods in section 2.5 to look for variation between months and years. Only GP surgeries that fall within the geographical catchment of the sampled wastewater treatment facility were included for this study. For each compound included in the study, the monthly prescription data for the whole catchment area was used to calculate the average amount prescribed each day for that month (kg day^{-1}). The daily averages for each month were then used to calculate the daily average for the whole year. The average daily prescription for each year was then used to calculate the population normalised daily prescription (PNDP) of compound X using equation six (eq. 6).

$$PNDP_x(mg\ day^{-1} 1000\ inh^{-1}) = (Daily\ average)_x * \frac{1000}{population} * 10^6 \quad eq. 6$$

The PNDP of each compound was then comparable to the PNDL and daily intake of that compound calculated from wastewater. A limitation of this approach is that compares monthly prescription data to daily wastewater data, which may hid any short term changes in prescription rates that may be picked up in wastewater. Likewise, there is the assumption that everything is prescribed will be taken during the same period, which may not be true for medication like antihistamines or painkillers that are taken in response to an outside stimulus, like high pollen counts, rather than every day. An additional consideration is the assumption that prescriptions from GPs within the catchment will be consumed within the catchment, and vice-versa that only compounds prescribed from within the catchment will be consumed. As discussed with morphine in section 2.3 this could potentially result in an underestimation and an overestimation of consumption, respectively, but is not expected to be significant compared to other sources of error.

3. **Results and discussion**

3.1 Trends in average daily intake

The simplest way of viewing the results would be to examine the changes in average daily intake from 2014 to 2018 (Tables S3 and S4). Viewed this way four compounds had a significant decrease ($P<0.01$) in concentration (heroin, mephedrone, methadone and tramadol), two had no significant change in concentration ($P>0.05$) (amphetamine and codeine), and twelve had a significant increase ($P<0.01$) (cocaine, methamphetamine, MDMA, ketamine, hydrocodone, oxymorphone, morphine, temazepam, oxazepam, venlafaxine, ephedrine and pseudoephedrine). However, this simplistic view conceals a lot of important information on how daily intake changed over the study period.

Firstly, cocaine was the only compound to have a significant ($P<0.05$) year on year increase each year. As shown in Figure 1 the daily intake of cocaine roughly quadrupled from 579 ± 50 mg day^{-1} 1000 inh^{-1} in 2014 to 2258 ± 67 mg day^{-1} 1000 inh^{-1} in 2018. Two other compounds had a similarly increased daily intake from 2014-2018, with significant increases only reported for some years: oxazepam and pseudoephedrine (Figure 2). Average daily intake of oxazepam only increased significantly ($P<0.01$) between 2016 and 2017, whilst for pseudoephedrine the only increase ($P<0.05$) came between 2014 and 2015. In contrast, the average daily intake of codeine increased significantly ($P<0.05$) from 2017-2018 despite no significant overall increase in consumption from 2014-2018 ($P>0.05$).

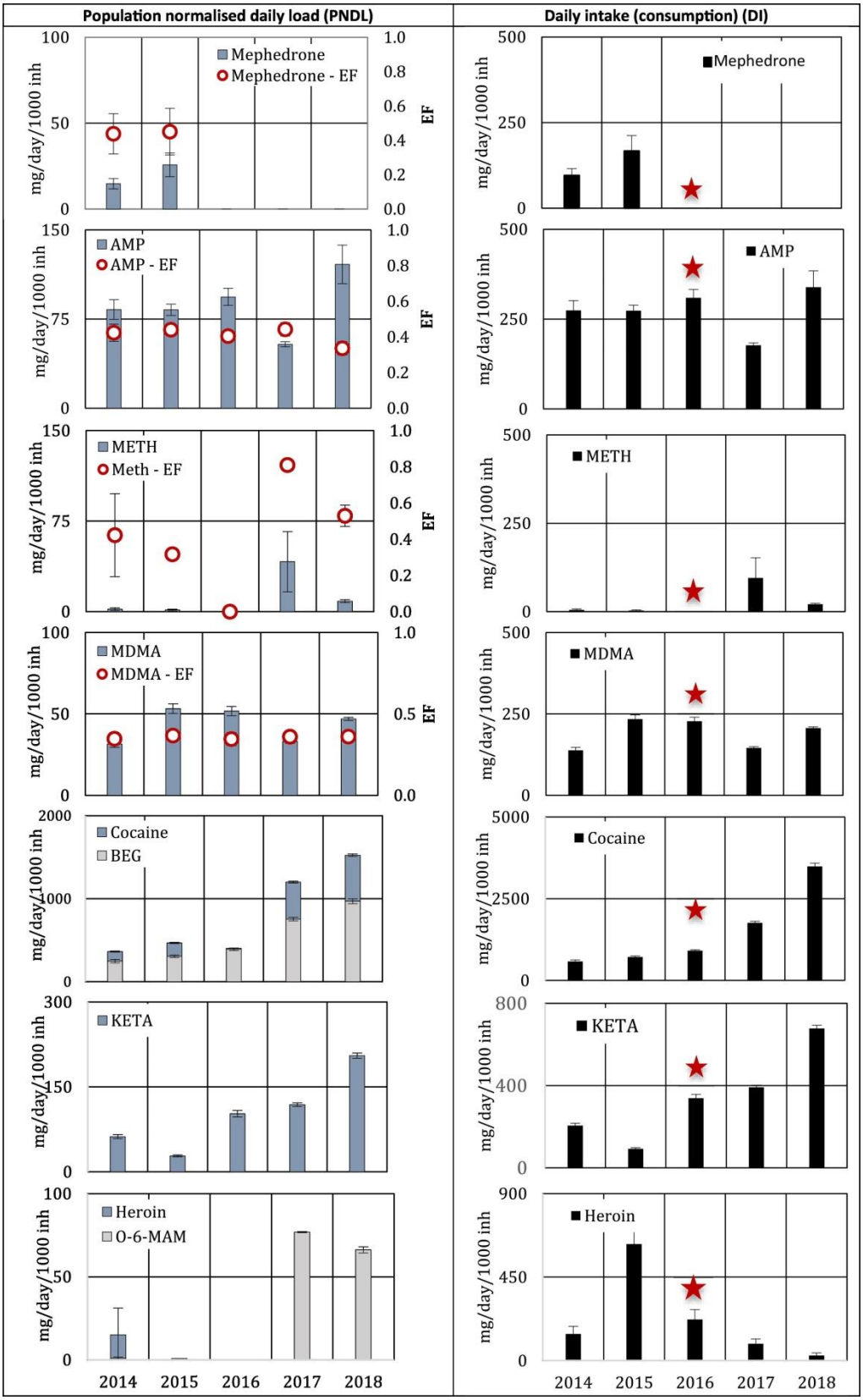


Figure 1. Average PNDLs and DIs of illicit drugs (2014-18) (star indicates 2016 NPS bill)

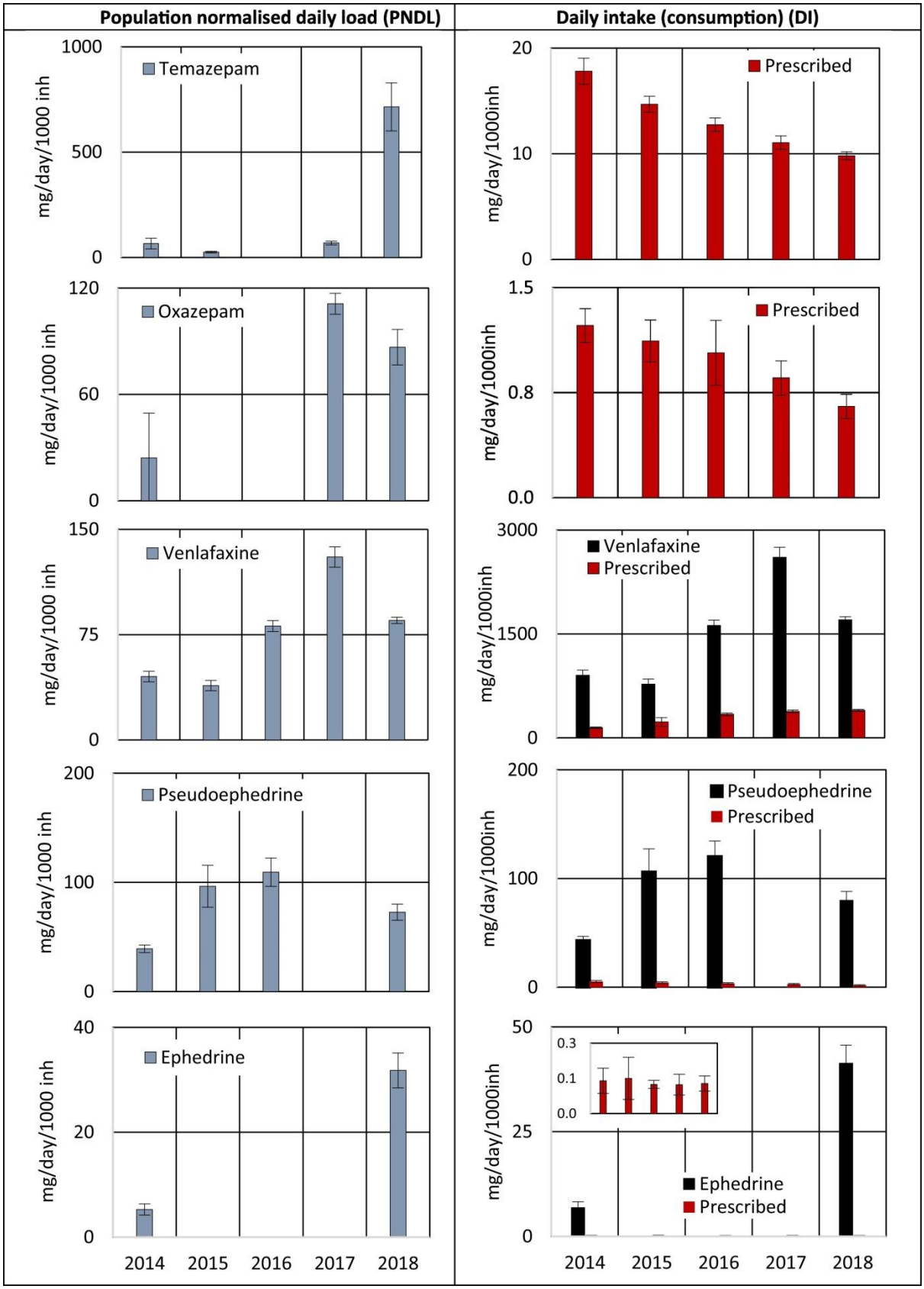


Figure 2. Average PNDLs, DIs and prescription of non-opioid pharmaceuticals (2014-18)

Cocaine exhibited a pronounced weekend effect each year, with significantly greater consumption ($P<0.01$) on weekend days (Friday-Monday) compared to weekday days (Tuesday-Thursday). Interestingly the increase in consumption between years was not necessarily equally divided across the week, with no significant increase ($P>0.05$) in weekday cocaine consumption between 2015 and 2016 despite an increase in average daily consumption between the two years. Only two others compounds also exhibited a pronounced weekend effect for the majority of years they were detected: ketamine and MDMA.

The average daily intake of MDMA was more complicated to understand than cocaine's, as despite significant increases ($P<0.01$) in average daily intake between 2014-2015 and 2017-2018 there was also a significant decrease ($P<0.01$) from 2016-2017 and overall there was no significant variation between years according to ANOVA ($P>0.05$). This apparent contradiction in MDMA consumption can be explained by its significant ($P<0.01$) and large weekend effect, which meant the data was not normally distributed making ANOVA unsuitable for assessing variation between years. Instead the average weekday and weekend daily intake were used to determine trends in MDMA consumption. Weekend and weekday average daily intake of MDMA both increased significantly ($P<0.01$) from 2014-2015 and 2017-2018, in line with the above observations. Additionally, there was then significant variation between years by ANOVA ($P<0.05$). From 2015-2016 however there was a significant decrease in weekday average daily intake ($P<0.01$), whilst weekend intake remained constant ($P>0.05$). This decrease was not previously captured when looking at MDMA consumption for the whole week. Similarly, from 2016-2017 there was a significant ($P<0.01$) decrease in weekend average daily intake and an insignificant ($P>0.05$) decrease in weekday average daily intake, resulting in the significant decrease in MDMA consumption detailed above. The overall increase in MDMA consumption from 2014-2018 was driven by a significant increase in the average daily weekend intake of MDMA, which increased from 185 ± 24 mg day^{-1} 1000 inh^{-1} in 2014 to 306 ± 12 mg day^{-1} 1000 inh^{-1} in 2018 whilst the average daily weekday intake did not vary significantly ($P>0.05$).

Meanwhile ketamine consumption was more similar to cocaine than MDMA, with a less pronounced weekend effect. Ketamine average daily intake increased each year from 2015-2018 and each of these years also exhibited a significant weekend effect ($P<0.05$). However, like with cocaine, the year on year increase was not equally distributed between weekend and weekday consumption with no significant increase ($P>0.05$) in weekend consumption of

ketamine between 2016 and 2017 despite an overall increase in average daily intake. Unlike cocaine and MDMA, ketamine is used medicinally, so some of this consumption could be the result of licit consumption. However, the presence of a weekend effect in most years suggested that it was likely being used recreationally.

The most common trend in average daily intake was exemplified by temazepam (Figure 2), which had a significant decrease ($P<0.01$) in year on year daily intake from 2015-2016, where it was <LOQ in 2016, and then significant increases ($P<0.01$) between 2016-2017 and 2017-2018 and no weekend effect. Oxymorphone and hydrocodone (Figure 3) both had similar trends in average daily intake, with an initial significant decrease ($P<0.05$) in consumption from 2015-2016, below LOQ PNDLs in 2016 and then a significant increase in consumption from 2016-2018 ($P<0.01$), leading to an overall increase in average daily intake from 2014-2018. Temazepam and hydrocodone also had a significant increase ($P<0.01$) in average daily intake between 2017 and 2018. Hydrocodone is a metabolite of codeine (Barakat, Atayee et al. 2012) as well as a prescription opioid in its own right. As codeine and hydrocodone had different overall trends in PNDL this suggested that the hydrocodone being detected resulted from hydrocodone consumption rather than codeine metabolism. For all these compounds the trend in consumption suggested that they were overall becoming more widely used despite an initial period of decreasing or variable use. In contrast, morphine (Figure 4) and venlafaxine (Figure 2), which also had an overall increase in daily intake from 2014-2018, behaved oppositely with an initial significant increase ($P<0.01$) in daily intake followed by a significant decrease ($P<0.01$). This suggested that usage of morphine and venlafaxine were then decreasing after an initial period of increased consumption.

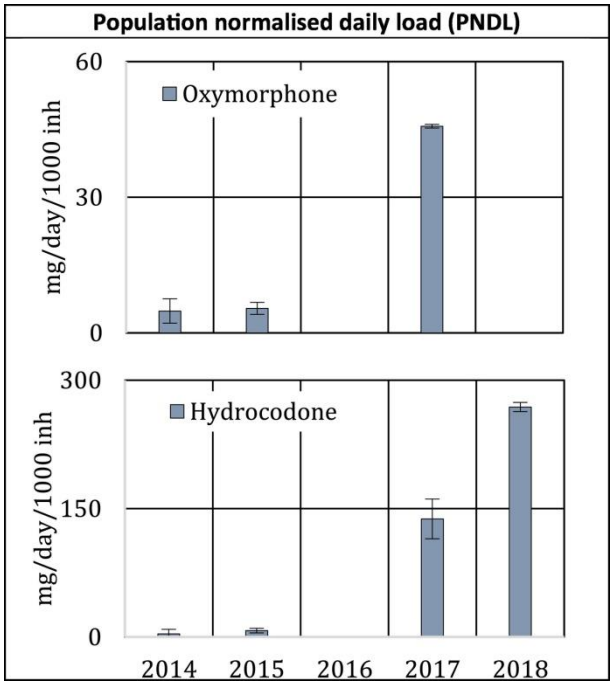


Figure 3. Average PNDLs of oxymorphone and hydrocodone (2014-18)

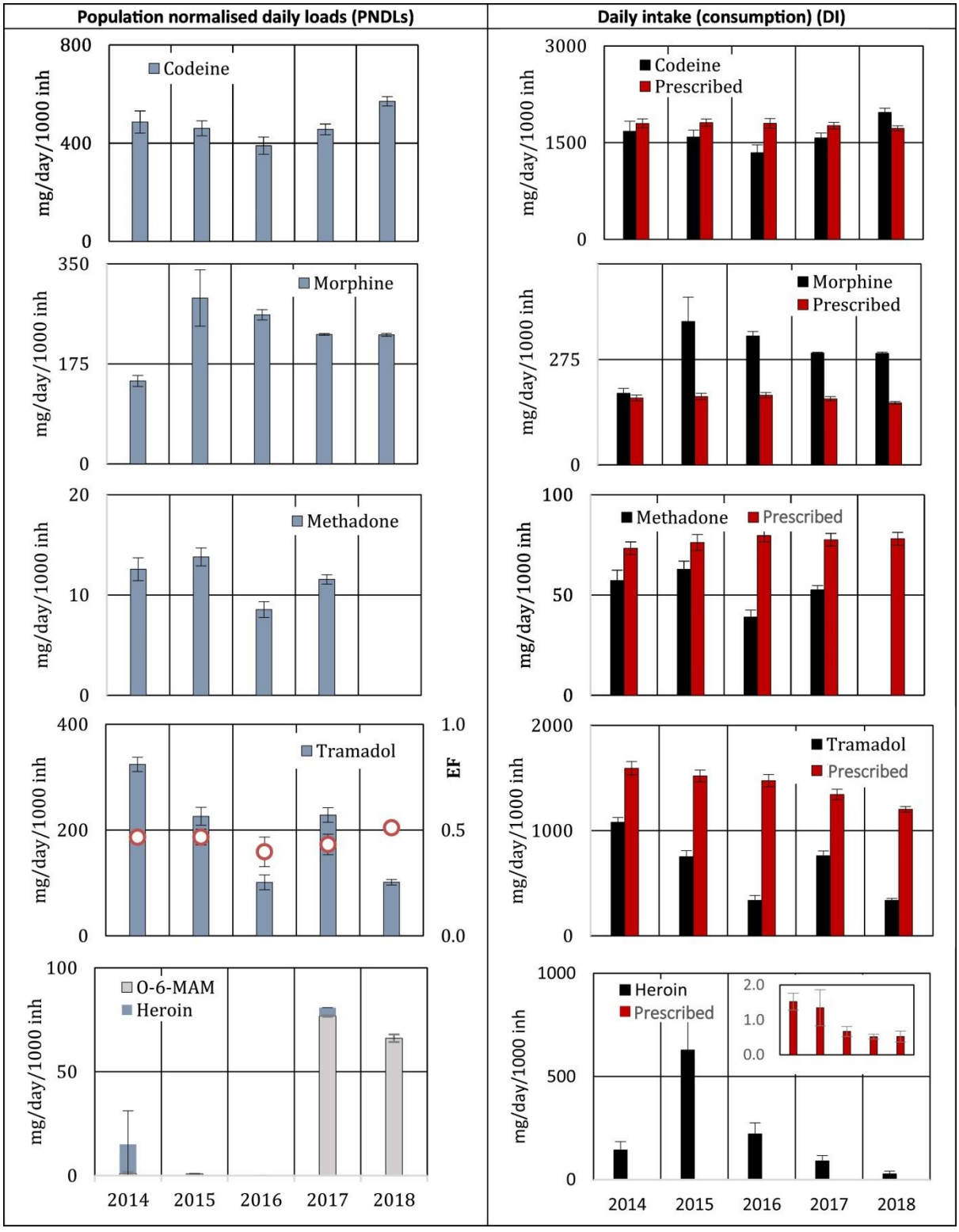


Figure 4. Average PNDLs, DIs and prescription of opioid pharmaceuticals (2014-18)

Tramadol had a pattern of alternating consumption with tramadol consumption significantly decreasing ($P<0.01$) from 2014-2018 and between every year except from 2016-2017. The EF of tramadol increased over the study period with a significant increase ($P<0.01$) from

2017-2018, despite a significant decrease from ($P<0.01$) 2015-2016, resulting in a racemic mixture of both enantiomers in 2018. Similar to tramadol, methadone exhibited an overall decrease with alternating trends in intake with a year decrease between 2015-2016 and a year on year increase from 2016-2017. Regrettably methadone's deuterated internal standard was not detected in 2018 samples so methadone could not be quantified for this year of the study, although it was detected. Despite an overall downwards trend in consumption across the study period, the alternating changes in daily intake suggested that consumption of these compounds was in a state of flux. Further monitoring and non-wastewater data sources would be necessary to see if this state of flux will continue or develop into broader trends. Heroin had a simpler pattern of consumption with average daily intake decreasing significantly ($P<0.01$) from 2014-2018 and between every year ($P<0.05$) except between 2014 and 2015 where there was a significant increase ($P<0.05$) in average daily intake. This supports other observations that the number of injectable drug users was decreasing (EMCDDA 2018) to suggest heroin was becoming a less popular drug of abuse. Despite having a similar pattern of consumption as morphine, which was used along with codeine to calculate heroin consumption, the two compounds behaved different overall with morphine consumption increasing significantly ($P<0.01$) from 2014-2018 whilst heroin consumption decreased significantly ($P<0.01$) over the same period. In contrast, if heroin daily intake was calculated using O-6-MAM then the daily intake was observed to increase significantly ($P<0.01$) between 2014-2018 with an alternating pattern of consumption similar to temazepam, oxazepam and hydrocodone. In this case the calculation of heroin consumption using morphine and codeine is likely more accurate than the calculation using O-6-MAM, particularly as the greatest O-6-MAM PNDLs occurred in 2017-2018 when heroin consumption from morphine was at its lowest level. Furthermore, using O-6-MAM lead to estimating heroin loads of >5700 mg day^{-1} 1000 inh^{-1} in 2017 and 2018, which is roughly 140-300% greater than consumption of cocaine, amphetamine, methamphetamine and MDMA combined in the same year and is therefore extremely unlikely to be accurate, particularly as heroin is meant to be less popular than cocaine in the UK (EMCDDA 2018).Therefore, further work is needed to accurately determine heroin consumption as the use of O-6-MAM seems to lead to an overestimation, and the approach of using morphine and codeine prescription and wastewater data (Du, Zhou et al. 2017) is still relatively novel and requires further work to validate its suitability and robustness.

Overall there was no significant change in amphetamine consumption ($P>0.05$) between 2014 and 2018, with the only significant change during the period being a significant decrease ($P<0.01$) in average daily intake between 2016 and 2017 and significant variation by ANOVA ($P<0.01$). The EF of amphetamine was similarly stable across the period of 2014-2018, with a significant increase from 2014-2015 ($P<0.05$) and a significant decrease ($P<0.05$) from 2017-2018 resulting in no net change in EF. The only year with a significant weekend effect ($P<0.01$) was 2017, where the decrease in weekday average daily intake of amphetamine was greater than the decrease in weekend average daily intake. Therefore, amphetamine consumption seemed very stable across the study period.

Lastly, the absence of mephedrone (Figure 1) from 2016 onwards and its static average daily intake from 2014-2015 suggested that it was no longer being widely consumed five years after first being regulated as a Class B drug of abuse. For the two years it was detected there was no significant change ($P>0.05$) in EF or any evidence of a weekend effect for mephedrone. The continued use of mephedrone after regulation was expected, as a survey of users said they were planning on continuing to use mephedrone (Winstock, Mitcheson et al. 2010). In contrast, other NPS that were regulated in the same manner and at the same time as mephedrone, such as synthetic cannabinoids, usually disappeared relatively quickly and were replaced with a structural analogue (Bijlsma, Ibanez et al. 2017). Mephedrone can be viewed as evidence of successful, if delayed, drug policy and, although not successful with all drugs of abuse, showed that regulation can lead to a cessation of consumption.

3.2 The use of prescription data

NHS prescription data was available for roughly half of the analytes detected, which allowed for greater insight into the trends discussed above. This was particularly useful for assessing opioids as there were disparity in trends of average daily intake of heroin (decreasing) and morphine (increasing). Codeine displayed excellent agreement between wastewater and prescription data (Figure 4), whilst tramadol and methadone displayed similar trends of decreasing average daily intake in wastewater and decreasing prescription rates. In both cases the wastewater concentration of tramadol and methadone were lower than the prescription data would suggest. Whilst most commonly associated with the treatment of opioid addiction, methadone is also prescribed for treating chronic pain, as is tramadol and as such they may be taken in response to pain rather than continuously. This could result in a lower wastewater

concentration as not all the tramadol or methadone prescribed will be consumed if it is being used periodically as part of chronic pain management.

In contrast venlafaxine and pseudoephedrine (Figure 2) had higher wastewater concentrations than would be expected from prescriptions, although both prescription and wastewater concentrations underwent the same trend of decreasing or stable consumption, respectively. Pseudoephedrine is available without a prescription or "over the counter" from a pharmacist and this may explain its relatively greater wastewater concentration. Alternatively, this could be evidence of the use of pseudoephedrine in clandestine manufacture of methamphetamine (Pal, Megharaj et al. 2012). However, this is unlikely as methamphetamine in Europe is not generally produced in the UK and methamphetamine is not a popular drug in the UK, which suggests low demand (EMCDDA 2019). As an antidepressant venlafaxine is not available in the UK without a prescription, however its availability online and associated abuse has been reported previously (Francesconi, Orsolini et al. 2015). Rates of antidepressant prescription were rising in the decades prior to the study period and were expected to continue increasing (Mars, Heron et al. 2017). The UK has been experiencing a period of political economic austerity since 2010 including across the period of 2014-2018, and under similar circumstances in other European countries this has led to an increase in prescriptions of antidepressants and other drugs to treat mental health (Thomaidis, Gago-Ferrero et al. 2016). In line with expectations venlafaxine prescription rates did increase significantly each year ($P<0.01$), although prescription rates of other antidepressants (amitriptyline and fluoxetine) did not change significantly ($P>0.05$) from 2014-2018 (Table S4). Increasing demand for antidepressants coupled with the online availability of venlafaxine and its relatively lower cost, compared to NHS prescriptions, could explain the mismatch between prescription and wastewater data. Ultimately, the reason behind the discrepancy in wastewater and prescription data is unclear, but what is important is that the trends in both of them are the same, which helps to provide important context to the results.

Of greater concern were those compounds for which prescription rates were decreasing from 2014-2018 but wastewater concentrations were increasing: morphine, temazepam and oxazepam. There was a significant difference between wastewater and prescription concentrations of morphine ($P<0.01$). However, this could have been the result of morphine production from opioid metabolism, such as codeine and heroin, although the discrepancy is only around 50 mg day^{-1} 1000 inh^{-1}. Prescription rates of diamorphine (figure 4) were low and stable throughout the study period in contrast to large decreases in the average daily

intake of heroin, but as it is a drug of abuse it was not surprising that diamorphine prescription would not match wastewater concentrations. Temazepam and oxazepam (Figure 2) prescriptions rates were a lot lower than their wastewater concentrations. This was likely due to the interconversion of benzodiazepines during metabolism, where oxazepam is a metabolite of temazepam, which is in turn a metabolite of diazepam (Whirl-Carrillo, McDonagh et al. 2012). From prescription data diazepam also had significantly decreasing ($P<0.01$) rates of prescription. Additionally, prescription of nitrazepam and lorazepam also decreased significantly ($P<0.01$) across the study period suggesting that all benzodiazepines were being prescribed less not just those detected in wastewater. It is important to note that nitrazepam and lorazepam have a different metabolic pathway to the other benzodiazepines discussed here. Benzodiazepines can be purchased online, which would result in prescription data underestimating consumption, however a review of availability in Europe suggested that prescriptions were the main source of benzodiazepines for non-medical use in the UK (Lyphout, Yates et al. 2019). Overall this suggested that benzodiazepines were being abused in greater quantities from 2016-2018, but the magnitude and source of this abuse was difficult to quantify. Prescription data was not available for other opioids with similar trends (oxymorphone and hydromorphone).

Whilst not detected above LOQ for most of the study period comments can be made about the use of Z-drugs and PDE-5 inhibitors from their prescription data. Prescription rates of sildenafil (Viagra) increased significantly ($P<0.05$) each year from 2014-2018, whilst prescription rates of the vardenafil (Levitra) decreased significantly each year ($P<0.05$), except from 2015-2016. Zopiclone prescription rates decreased each year and zolpidem prescription rates decreased between 2014-2015 and 2017-2018. As Z-drugs are pharmacologically similar to benzodiazepines it seemed logical that there would be similar trends in how they were prescribed.

3.3 A European perspective

A recent publication of European wastewater data covering the period of 2011-2017 provided crucial context for the result of this study (Gonzalez-Marino, Baz-Lomba et al. 2020), with additional data for 2018 available online from the EMCDDA. The European study reported the average daily intake of benzoylecgonine (cocaine), amphetamine, methamphetamine and MDMA over a period of one week for several European cities (Figure 5) with our UK city

contributing data from 2014-2018. In 2011 the most abundant drugs were amphetamine and cocaine, followed by methamphetamine and MDMA, which also had similar abundance.

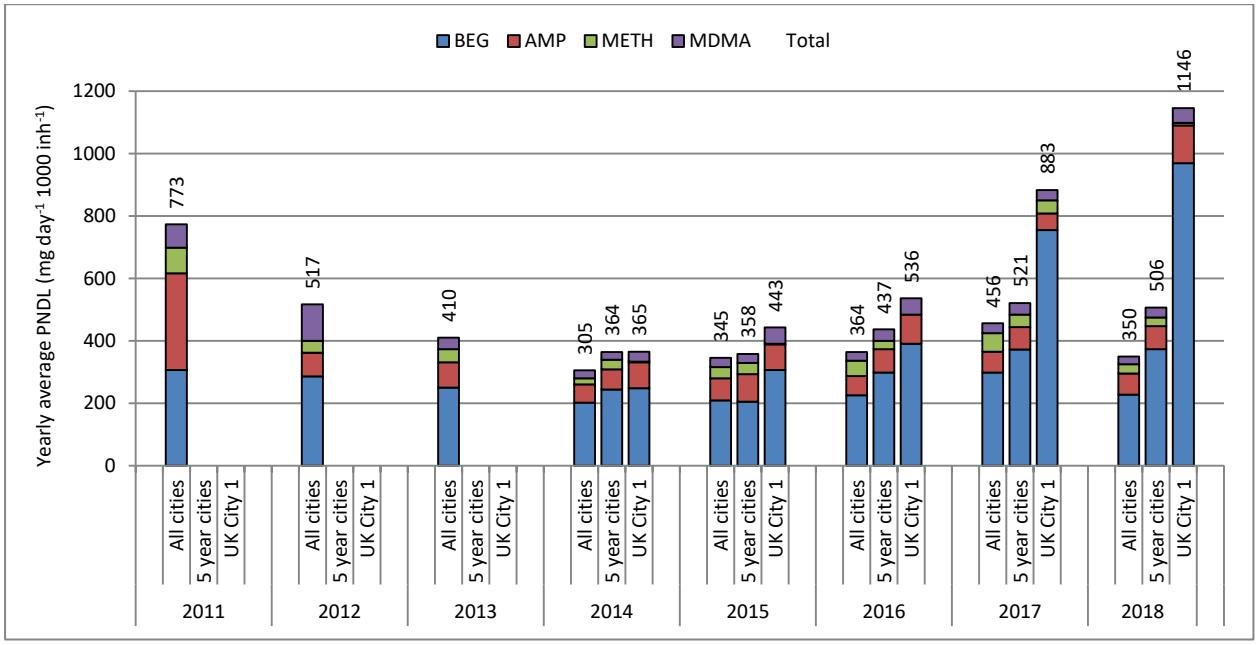


Figure 5. Average PNDL in Europe of benzoylecgonine (BEG), amphetamine (AMP), methamphetamine (METH) and ecstasy (MDMA) (2011-2018). All cities refers to all cities that contributed to the study that year, 5 year cities refers to cities that contributed to the study each year from 2014-2018, UK City 1 is the city studied in this manuscript.

From 2011-2014 the sum total of all four analytes decreased by ≈60 % and the relative abundance of cocaine increased from ≈40% to ≈66% of the total drug load. From 2014-2018 the total drug load and % BEG increased reaching a maximum in 2017 before decreasing slightly in 2018 to approximately the same levels as 2016. If only the cities that contributed every year from 2014-2018, excluding our study city, (5-year cities) are included then the total drug load and % BEG continued to increase in 2018. Overall the paper noted several trends exhibited in European drug use:

1) Cocaine use increased year on year
2) MDMA consumption increased intermittently over the period of 2014-2018
3) Amphetamine use was only increasing in Northern Europe, Belgium and the Netherlands
4) Methamphetamine use was mostly limited to Central and Eastern Europe

This helped to provide context for illicit drug consumption in our study city. Firstly, whilst cocaine usage was increasing in Europe it was increasing faster in the UK, as evidenced by a much greater % BEG compared to the 5-year cities from 2015 onwards, despite a very similar % BEG in 2014. This suggested that there were UK specific factors driving the increase in cocaine consumption, causing it to increase at a greater rate than in Europe. Conversely sequential year t-tests of weekday and weekend MDMA consumption showed that there was a significant increase ($P<0.01$) in the use of MDMA in the UK across the study period, which agreed with European observations. Additionally the source of MDMA in the UK appeared to be from consumption rather than from disposal or manufacturing, which has been observed in Dutch cities, as the EFs of MDMA were consistent ($P>0.05$) and non-racemic (Emke, Evans et al. 2014). Additionally, UK drug consumption of amphetamine and methamphetamine matched expected spatial trends within Europe with amphetamine usage remaining constant, and low but increasing usage of methamphetamine, which was the least abundant of these four drugs of abuse.

3.4 UK specific trends in consumption

European data on cocaine, amphetamine, methamphetamine and MDMA usage showed that drug consumption was decreasing from 2011-2014 and then increased slowly before appearing to stabilise in 2018. However, for the UK the rate of total drug consumption increased significantly ($P<0.01$) over the period of 2014-2018 and continued increasing instead of stabilising. In 2016 the NPS bill was brought into force, banning the use of all novel psychoactive substance except those compounds either covered by existing regulations, such as medicines or drugs of abuse, or specifically exempted, like caffeine or nicotine. The majority of compounds analysed as part of this manuscript exhibited a decrease in average daily intake from 2014-2016, i.e. before the bill came into law, but then exhibited an increase in average daily intake from 2016-2018.

Venlafaxine, pseudoephedrine, codeine, heroin, cocaine, amphetamine and MDMA did not exhibit a significant decrease ($P<0.05$) from 2014-2016, with all except amphetamine, codeine and heroin actually undergoing a significant increase ($P<0.01$) in consumption. Then from 2016-2018 only amphetamine, morphine, heroin and pseudoephedrine did not experience a significant increase ($P<0.05$) in average daily intake, with morphine, heroin and pseudoephedrine consumption decreasing significantly ($P<0.01$). The volume of wastewater used in the study did decrease from 100 mL to 50 mL between 2014 and 2015, and for some

compounds with low abundance, such as methamphetamine, this could explain their apparent decrease. However, this would still not explain why concentrations of so many analytes increased after 2016 when the population of our city had not changed.

Whilst it is still too early to assess the impact of the 2016 NPS bill, particularly as this study did not try and analyse contemporary NPS such as synthetic cannabinoids, the observed increase in drug consumption was concerning. Heroin, methadone and mephedrone were the only compounds to have a significantly lower ($P<0.01$) average daily drug intake after 2016, whilst consumption of amphetamine and tramadol did not significantly change ($P>0.05$). None of the drugs of abuse included in this study were regulated any more heavily over the study period, so a potential reason for the increased consumption is that drug users migrated from NPS to more "popular" drugs of abuse such as cocaine and ketamine, both of which saw their average daily intake quadruple across the study period. This was also observed by the UK government in its review of the 2016 NPS bill and concluded that there was not enough data to support this (Home Office, 2018), although this review did not include an assessment of wastewater. Whilst no conclusive this report does support the observations that there has been an increase in Class A drug use. Further work would be necessary to determine if this effect was UK wide as previous studies of other countries have revealed differences in drug consumption depending on city size and socioeconomic factors (Been, Lai et al. 2016, Choi, Tscharke et al. 2019).

The agreement between wastewater and prescription data concerning decreasing consumption of tramadol and largely unchanged consumption of codeine, were positive as, at time of writing, other countries, like the United States of America, were combatting an ongoing opioid epidemic centred on abuse of prescription opioids (Cicero, Ellis et al. 2015, Champagne-Langabeer, Madu et al. 2019, Cicero, Ellis et al. 2020). Likewise, decreasing consumption of methadone combined with decreasing consumption of heroin was positive. Available non-wastewater data from the EMCDDA showed that the number of heroin users, as well as the number of injectable drug users in general, was decreasing across the study period (EMCDDA 2018). Available data on heroin pricing and purity in Europe showed that both purity and cost had dropped by 13 % and 20 % respectively from 2007-2017 (European Monitoring Centre for Drugs and Drug Addiction and Europol 2019). Whilst this decrease in price could be expected to lead to an increase in heroin users, available data suggested that heroin was price inelastic (Olmstead, Alessi et al. 2015) and as such demand would not be expected to increase, which matched what was observed in wastewater.

4. Conclusions

Consumption of illicit drugs of abuse has generally increased from 2014 to 2018 particularly for cocaine, methamphetamine and ketamine and this cannot purely be explained by either wider European drug trends, or increased prescription of licit forms of the drug. Likewise, increasing consumption of cocaine in the wake of the 2016 NPS bill could not be purely explained by increasing consumption across Europe.

The benefits of combining unique prescription and wastewater data were shown by the ability of both to explain apparently misleading trends identified in one another. This was particularly beneficial for compounds with pharmaceutically active metabolites like opioids and for identifying sources of morphine to estimate heroin consumption. Additionally, for compounds with controlled prescription like methadone, trends in consumption by wastewater and trends in prescription correlated to show that there is a link between what is prescribed and what is consumed. This shows that wastewater-based epidemiology is a powerful tool for examining whole populations and determining the efficacy and direction of government actions on health, as it can, alongside prescription and wider monitoring data, provide a clear insight into what is being consumed by a population and what action is needed to meet required goals.

Acknowledgments

The support of Wessex Water Services Ltd and the University of Bath EPSRC Impact Acceleration Account (Project number: EP/ K503897/1 and EP/R51164X/1) is greatly appreciated. This work was also supported by the European Union's Seventh Framework Programme for research, technological development and demonstration [Grant agreement 629015, the MC IEF project 'Chiral veterinary medicines in the environment'] [grant agreement 317205, the SEWPROF MC ITN project, 'A new paradigm in drug use and human health risk assessment: Sewage profiling at the community level'] and the Leverhulme Trust (Project No RPG-2013-297).

References:

EMCDDA. (2018). United Kingdom, Country Drug Report 2018**:** 1-26.
Baker, D. R., L. Barron and B. Kasprzyk-Hordern (2014). "Illicit and pharmaceutical drug consumption estimated via wastewater analysis. Part A: Chemical analysis and drug use estimates." Science of the Total Environment **487**: 629-641.
Barakat, N. H., R. S. Atayee, B. M. Best and A. J. Pesce (2012). "Relationship between the Concentration of Hydrocodone and its Conversion to Hydromorphone in Chronic Pain Patients Using Urinary Excretion Data." Journal of Analytical Toxicology **36**(4): 257-264.
Baz-Lomba, J. A., S. Salvatore, E. Gracia-Lor, R. Bade, S. Castiglioni, E. Castrignano, A. Causanilles, F. Hernandez, B. Kasprzyk-Hordern, J. Kinyua, A. K. McCall, A. van Nuijs, C. Ort, B. G. Plosz, P. Ramin, M. Reid, N. I. Rousis, Y. Ryu, P. de Voogt, J. Bramness and K. Thomas (2016). "Comparison of pharmaceutical, illicit drug, alcohol, nicotine and caffeine levels in wastewater with sale, seizure and consumption data for 8 European cities." Bmc Public Health **16**.
Been, F., F. Y. Lai, J. Kinyua, A. Covaci and A. L. N. van Nuijs (2016). "Profiles and changes in stimulant use in Belgium in the period of 2011-2015." Science of the Total Environment **565**: 1011-1019.
Bijlsma, L., M. Ibanez, B. Miserez, S. T. F. Ma, T. Shine, J. Ramsey and F. Hernandez (2017). "Mass spectrometric identification and structural analysis of the third-generation synthetic cannabinoids on the UK market since the 2013 legislative ban." Forensic Toxicology **35**(2): 376-388.
Castiglioni, S., L. Bijlsma, A. Covaci, E. Emke, C. Harman, F. Hernandez, B. Kasprzyk-Hordern, C. Ort, A. L. N. van Nujis, P. de Voogt and E. Zuccato (2016). Estimating community drug use through wastewater-based epidemiology. Assessing illict drugs in wastewater: advances in wastewaster-based epidemiology, Publications office of the European Union, Luxembourg**:** 17-34.
Castiglioni, S. and E. Gracia Lor (2016). Target drug residues in wastewater. Assessing illicit drugs in wastewater: advances in wastewater-based drug epidemiology. S. Castiglioni. Publications office of the European Union, Luxembourg, EMCDDA**:** 35-45.
Castrignano, E., A. Lubben and B. Kasprzyk-Hordern (2016). "Enantiomeric profiling of chiral drug biomarkers in wastewater with the usage of chiral liquid chromatography coupled with tandem mass spectrometry." Journal of Chromatography A **1438**: 84-99.
Castrignano, E., Z. Yang, R. Bade, J. A. Baz-Lomba, S. Castiglioni, A. Causanilles, A. Covaci, E. Gracia-Lor, F. Hernandez, J. Kinyua, A.-K. McCall, A. L. N. van Nuijs, C. Ort, B. G. Plosz, P. Ramin, N. I. Rousis, Y. Ryu, K. V. Thomas, P. de Voogt, E. Zuccato and B. Kasprzyk-Hordern (2018). "Enantiomeric profiling of chiral illicit drugs in a pan-European study." Water Research **130**: 151-160.
Champagne-Langabeer, T., R. Madu, S. Giri, A. L. Stotts and J. R. Langabeer (2019). "Opioid prescribing patterns and overdose deaths in Texas." Substance Abuse.
Choi, P. M., B. Tscharke, S. Samanipour, W. D. Hall, C. E. Gartner, J. F. Mueller, K. V. Thomas and J. W. O'Brien (2019). "Social, demographic, and economic correlates of food and chemical consumption measured by wastewater-based epidemiology." Proceedings of the National Academy of Sciences of the United States of America **116**(43): 21864-21873.
Cicero, T. J., M. S. Ellis and J. Harney (2015). "Shifting Patterns of Prescription Opioid and Heroin Abuse in the United States." New England Journal of Medicine **373**(18): 1789-1790.
Cicero, T. J., M. S. Ellis and Z. A. Kasper (2020). "Polysubstance Use: A Broader Understanding of Substance Use During the Opioid Crisis." American journal of public health **110**(2): 244-250.
Du, P., Z. L. Zhou, Y. Bai, Z. Q. Xu, T. T. Gao, X. F. Fu and X. Q. Li (2017). "Estimating heroin abuse in major Chinese cities through wastewater-based epidemiology." Science of the Total Environment **605**: 158-165.
EMCDDA (2018). United Kingdom Country Drug Report 2018.
EMCDDA, E. M. C. f. D. a. D. A. (2019). European Drug report 2019: Trends and developments. Luxembourg, Publications Office of the European Union.
EMCDDA, E. M. C. f. D. a. D. A. (2020). Wastewater analysis and drugs: a European multi-city study.

Emke, E., S. Evans, B. Kasprzyk-Hordern and P. de Voogt (2014). "Enantiomer profiling of high loads of amphetamine and MDMA in communal sewage: A Dutch perspective." Science of the Total Environment **487**: 666-672.
European Monitoring Centre for Drugs and Drug Addiction and Europol (2019). EU drug markets report 2019. Luxembourg, Publications office of the European Union.
Francesconi, G., L. Orsolini, D. Papanti, J. M. Corkery and F. Schifano (2015). "Venlafaxine as the "baby ecstasy'? Literature overview and analysis of web-based misusers experiences." Human Psychopharmacology-Clinical and Experimental **30**(4): 255-261.
Gonzalez-Marino, I., J. A. Baz-Lomba, N. A. Alygizakis, M. J. Andres-Costa, R. Bade, L. P. Barron, F. Been, J. D. Berset, L. Bijlsma, I. Bodik, A. Brenner, A. L. Brock, D. A. Burgard, E. Castrignano, C. E. Christophoridis, A. Covaci, P. de Voogt, D. A. Devault, M. J. Dias, E. Emke, D. Fatta-Kassinos, G. Fedorova, K. Fytianos, C. Gerber, R. Grabic, S. Gruner, T. Gunnar, E. Hapeshi, E. Heath, B. Helm, F. Hernandez, A. Kankaanpaa, S. Karolak, B. Kasprzyk-Hordern, I. Krizman-Matasic, F. Y. Lai, W. Lechowicz, A. Lopes, M. L. de Alda, E. Lopez-Garcia, A. S. C. Love, N. Mastroianni, G. L. McEneff, R. Montes, K. Munro, T. Nefau, H. Oberacher, J. W. O'Brien, K. Olafsdottir, Y. Pico, B. G. Plosz, F. Polesel, C. Postigo, J. B. Quintana, P. Ramin, M. J. Reid, J. Rice, R. Rodil, I. Senta, S. M. Simoes, M. M. Sremacki, K. Styszko, S. Terzic, N. S. Thomaidis, K. V. Thomas, B. Tscharke, A. L. N. van Nuijs, V. Yargeau, E. Zuccato, S. Castiglioni and C. Ort (2020). "Spatio-temporal assessment of illicit drug use at large scale: evidence from 7 years of international wastewater monitoring." Addiction **115**(1): 109-120.
Goulding, N., M. Hickman, M. Reid, E. J. Amundsen, J. A. Baz-Lomba, J. W. O'Brien, B. J. Tscharke, P. deVoogt, E. Emke, W. Kuijpers, W. Hall and H. E. Jones (2020). "A comparison of trends in wastewater-based data and traditional epidemiological indicators of stimulant consumption in three locations." Addiction **115**(3): 462-472.
Gracia-Lor, E., E. Zuccato and S. Castiglioni (2016). "Refining correction factors. for back-calculation of illicit drug use." Science of the Total Environment **573**: 1648-1659.
Home Office. (2015). "Psychoactive Substances Act 2016." Updated 18th November 2018. Retrieved 09 July, 2019.
Iversen, L., S. Gibbons, O. M. R. Eden and A. Milton (2011). Consideration of the Novel Psychoactive Substances ('legal highs')**:** 1-70.
Khan, U. and J. A. Nicell (2011). "Refined sewer epidemiology mass balances and their application to heroin, cocaine and ecstasy." Environment International **37**(7): 1236-1252.
Krizman, I., I. Senta, M. Ahel and S. Terzic (2016). "Wastewater-based assessment of regional and temporal consumption patterns of illicit drugs and therapeutic opioids in Croatia." Science of the Total Environment **566**: 454-462.
Lyphout, C., C. Yates, Z. R. Margolin, P. I. Dargan, A. M. Dines, F. Heyerdahl, K. E. Hovda, I. Giraudon, B. Bucher-Bartelson, J. L. Green, D. M. Wood and D. E. N. R. G. Euro (2019). "Presentations to the emergency department with non-medical use of benzodiazepines and Z-drugs: profiling and relation to sales data." European Journal of Clinical Pharmacology **75**(1): 77-85.
Mars, B., J. Heron, D. Kessler, N. M. Davies, R. M. Martin, K. H. Thomas and D. Gunnell (2017). "Influences on antidepressant prescribing trends in the UK: 1995-2011." Social Psychiatry and Psychiatric Epidemiology **52**(2): 193-200.
Home Office, UK. (2015). Guidance for local authorities on taking action against 'head shops' selling new psychoactive substances - working with local partners, UK Home Office**:** 1-14.
Home Office, UK. (2017). Drug Strategy 2017**:** 1-52.
Home Office, UK. (2018). Review of the Novel Psychoactive Substances act 2016.
Olmstead, T. A., S. M. Alessi, B. Kline, R. L. Pacula and N. M. Petry (2015). "The price elasticity of demand for heroin: Matched longitudinal and experimental evidence." Journal of Health Economics **41**: 59-71.

Ort, C., J. M. Eppler, A. Scheidegger, J. Rieckermann, M. Kinzig and F. Soergel (2014). "Challenges of surveying wastewater drug loads of small populations and generalizable aspects on optimizing monitoring design." Addiction **109**(3): 472-481.
Ort, C., A. L. N. van Nuijs, J.-D. Berset, L. Bijlsma, S. Castiglioni, A. Covaci, P. de Voogt, E. Emke, D. Fatta-Kassinos, P. Griffiths, F. Hernandez, I. Gonzalez-Marino, R. Grabic, B. Kasprzyk-Hordern, N. Mastroianni, A. Meierjohann, T. Nefau, M. Oestman, Y. Pico, I. Racamonde, M. Reid, J. Slobodnik, S. Terzic, N. Thomaidis and K. V. Thomas (2014). "Spatial differences and temporal changes in illicit drug use in Europe quantified by wastewater analysis." Addiction **109**(8): 1338-1352.
Pal, R., M. Megharaj, R. Naidu, G. Klass, M. Cox and K. P. Kirkbride (2012). "Degradation in soil of precursors and by-products associated with the illicit manufacture of methylamphetamine: Implications for clandestine drug laboratory investigation." Forensic Science International **220**(1-3): 245-250.
Postigo, C., M. Lopez de Alda and D. Barcelo (2011). "Evaluation of drugs of abuse use and trends in a prison through wastewater analysis." Environment International **37**(1): 49-55.
Reuter, P. and A. Stevens (2007). Bringing evidence and analysis together to inform UK drug policy, The UK Drug Policy Commission**:** 1-108.
Smith, H. S. (2009). "Opioid Metabolism." Mayo Clinic Proceedings **84**(7): 613-624.
Terzic, S., I. Senta and M. Ahel (2010). "Illicit drugs in wastewater of the city of Zagreb (Croatia) - Estimation of drug abuse in a transition country." Environmental Pollution **158**(8): 2686-2693.
Thai, P. K., J. W. O'Brien, B. J. Tscharke and J. F. Mueller (2019). "Analyzing Wastewater Samples Collected during Census To Determine the Correction Factors of Drugs for Wastewater-Based Epidemiology: The Case of Codeine and Methadone." Environmental Science & Technology Letters **6**(5): 265-269.
Thomaidis, N. S., P. Gago-Ferrero, C. Ort, N. C. Maragou, N. A. Alygizakis, V. L. Borova and M. E. Dasenaki (2016). "Reflection of Socioeconomic Changes in Wastewater: Licit and Illicit Drug Use Patterns." Environmental Science & Technology **50**(18): 10065-10072.
van Nuijs, A. L. N., A. Covaci, H. Beyers, L. Bervoets, R. Blust, G. Verpooten, H. Neels and P. G. Jorens (2015). "Do concentrations of pharmaceuticals in sewage reflect prescription figures?" Environmental Science and Pollution Research **22**(12): 9110-9118.
van Nuijs, A. L. N., J. F. Mougel, I. Tarcomnicu, L. Bervoets, R. Blust, P. G. Jorens, H. Neels and A. Covaci (2011). "A one year investigation of the occurrence of illicit drugs in wastewater from Brussels, Belgium." Journal of Environmental Monitoring **13**(4): 1008-1016.
van Wel, J. H. P., E. Gracia-Lor, A. L. N. van Nuijs, J. Kinyua, S. Salvatore, S. Castiglioni, J. G. Bramness, A. Covaci and G. Van Hal (2016). "Investigation of agreement between wastewater-based epidemiology and survey data on alcohol and nicotine use in a community." Drug and Alcohol Dependence **162**: 170-175.
Whirl-Carrillo, M., E. M. McDonagh, J. M. Herbert, L. Gong, K. Sangkuhk, C. F. Thorn, R. B. Altman and T. E. Klein (2012). "Pharmacogenomics knowledge for personalized medicine." Clinical pharmacology and therapeutics.
Winstock, A., L. Mitcheson and J. Marsden (2010). "Mephedrone: still available and twice the price." Lancet **376**(9752): 1537-1537.
Zuccato, E., C. Chiabrando, S. Castiglioni, R. Bagnati and R. Fanelli (2008). "Estimating community drug abuse by wastewater analysis." Environmental Health Perspectives **116**(8): 1027-1032.
Zuccato, E., C. Chiabrando, S. Castiglioni, D. Calamari, R. Bagnati, S. Schiarea and R. Fanelli (2005). "Cocaine in surface waters: a new evidence-based tool to monitor community drug abuse." Environmental health : a global access science source **4**: 14-14.

Team, R. C. (2013) R: A Language and Environment for Statistical Computing. *R Foundation for Statistical Computing: Vienna* (2009). Jagadeesan, K. and B. Kasprzyk-Hordern (Manuscript in preparation). "PDA: an interactive tool to visualise spatiotemporal trends of total prescribed quantity of active pharmaceutical ingredients for the year 2014 to 2018."

Baker, D. R., L. Barron and B. Kasprzyk-Hordern (2014). "Illicit and pharmaceutical drug consumption estimated via wastewater analysis. Part A: Chemical analysis and drug use estimates." Science of the Total Environment **487**: 629-641.

Castrignano, E., Z. G. Yang, R. Bade, J. A. Baz-Lomba, S. Castiglioni, A. Causanilles, A. Covaci, E. Gracia-Lor, F. Hernandez, J. Kinyua, A. K. McCall, A. L. N. van Nuijs, C. Ort, B. G. Plosz, P. Ramin, N. I. Rousis, Y. Ryu, K. V. Thomas, P. de Voogt, E. Zuccato and B. Kasprzyk-Hordern (2018). "Enantiomeric profiling of chiral illicit drugs in a pan-European study." Water Research **130**: 151-160.

Gonzalez-Marino, I., J. A. Baz-Lomba, N. A. Alygizakis, M. J. Andres-Costa, R. Bade, L. P. Barron, F. Been, J. D. Berset, L. Bijlsma, I. Bodik, A. Brenner, A. L. Brock, D. A. Burgard, E. Castrignano, C. E. Christophoridis, A. Covaci, P. de Voogt, D. A. Devault, M. J. Dias, E. Emke, D. Fatta-Kassinos, G. Fedorova, K. Fytianos, C. Gerber, R. Grabic, S. Gruner, T. Gunnar, E. Hapeshi, E. Heath, B. Helm, F. Hernandez, A. Kankaanpaa, S. Karolak, B. Kasprzyk-Hordern, I. Krizman-Matasic, F. Y. Lai, W. Lechowicz, A. Lopes, M. L. de Alda, E. Lopez-Garcia, A. S. C. Love, N. Mastroianni, G. L. McEneff, R. Montes, K. Munro, T. Nefau, H. Oberacher, J. W. O'Brien, K. Olafsdottir, Y. Pico, B. G. Plosz, F. Polesel, C. Postigo, J. B. Quintana, P. Ramin, M. J. Reid, J. Rice, R. Rodil, I. Senta, S. M. Simoes, M. M. Sremacki, K. Styszko, S. Terzic, N. S. Thomaidis, K. V. Thomas, B. Tscharke, A. L. N. van Nuijs, V. Yargeau, E. Zuccato, S. Castiglioni and C. Ort (2020). "Spatio-temporal assessment of illicit drug use at large scale: evidence from 7 years of international wastewater monitoring." Addiction **115**(1): 109-120.

Gracia-Lor, E., E. Zuccato and S. Castiglioni (2016). "Refining correction factors. for back-calculation of illicit drug use." Science of the Total Environment **573**: 1648-1659.

Khan, U. and J. A. Nicell (2011). "Refined sewer epidemiology mass balances and their application to heroin, cocaine and ecstasy." Environment International **37**(7): 1236-1252.

Postigo, C., M. Lopez de Alda and D. Barcelo (2011). "Evaluation of drugs of abuse use and trends in a prison through wastewater analysis." Environment International **37**(1): 49-55.

Yargeau, V., B. Taylor, H. Li, A. Rodayan and C. D. Metcalfe (2014). "Analysis of drugs of abuse in wastewater from two Canadian cities." Science of the Total Environment **487**: 722-730.

Supplementary information

Table S1. Selected analytes and their properties

Compound	CAS	Formula	MW	Supplier
1,7-dimethylxanthine	611-59-6	$C_7H_8N_4O_2$	180.2	Sigma-Aldrich
2-(3,4-Dihydroxyphenyl)-N-methylpropylamine (DHMA)	15398-87-5	$C_{10}H_{15}NO_2$	181.2	Kinesis
2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP)	30223-73-5	$C_{20}H_{23}N$	277.4	LGC (Cerilliant product)
3,4-methylenedioxy amphetamine (MDA)	4764-17-4	$C_{10}H_{13}NO_2$	179.2	LGC (Cerilliant product)
3,4-methylenedioxy ethylamphetamine (MDEA)	82801-81-8	$C_{12}H_{17}NO_2$	207.3	LGC (Cerilliant product)
3,4-methylenedioxy methamphetamine (MDMA)	42542-10-9	$C_{11}H_{15}NO_2$	193.2	LGC
6-acetylmorphine	2784-73-8	$C_{19}H_{21}NO_4$	327.4	Sigma Aldrich (Cerilliant product)
7-aminonitrazepam	4928-02-3	$C_{15}H_{13}N_3O$	251.3	Sigma Aldrich (Cerilliant product)
Amitriptyline	549-18-8	$C_{20}H_{23}N$	277.4	Sigma-Aldrich
Amphetamine	300-62-9	$C_9H_{13}N$	135.2	LGC (Cerilliant product)
Anhydroecgonine methyl ester	43021-26-7	$C_{10}H_{15}NO_2$	181.2	Sigma Aldrich (Cerilliant product)
Benzoylecgonine	519-09-5	$C_{16}H_{19}NO_4$	289.3	Sigma-Aldrich
Benzylpiperazine	2759-28-6	$C_{11}H_{16}N_2$	176.3	LGC
Caffeine	58-08-2	$C_8H_{10}N_4O_2$	194.2	Sigma-Aldrich
Cocaethylene	529-38-4	$C_{18}H_{23}NO_4$	317.4	Sigma Aldrich (Cerilliant product)
Cocaine	50-36-2	$C_{17}H_{21}NO_4$	303.4	LGC (Cerilliant product)
Codeine	76-57-3	$C_{18}H_{21}NO_3$	299.4	Sigma-Aldrich
Cotinine	486-56-6	$C_{10}H_{12}N_2O$	176.2	Sigma Aldrich (Cerilliant product)
Creatinine	60-27-5	$C_4H_7N_3O$	113.1	Sigma-Aldrich
d,l-4-Hydroxy-3-methoxy amphetamine (HMA)	13062-61-8	$C_{10}H_{15}NO_2$	181.2	Kinesis
d,l-4-Hydroxy-3-methoxy methamphetamine (HMMA)	438625-58-2	$C_{11}H_{17}NO_2$	195.2	Kinesis
Desmethylvenlafaxine	93413-62-8	$C_{16}H_{25}NO_2$	263.4	Sigma-Aldrich
Diazepam	439-14-5	$C_{16}H_{13}ClN_2O$	284.7	Sigma Aldrich (Cerilliant product)
Dihydrocodeine	125-28-0	$C_{18}H_{23}NO_3$	301.4	Sigma Aldrich (Cerilliant product)
Dihydromorphine	509-60-4	$C_{17}H_{21}NO_3$	287.4	Sigma Aldrich (Cerilliant product)
Ephedrine	50-98-6	$C_{10}H_{15}NO$	165.2	Sigma-Aldrich
Fluoxetine	59333-67-4	$C_{17}H_{18}F_3NO$	309.3	LGC (Cerilliant product)
Heroin	561-27-3	$C_{21}H_{23}NO_5$	369.4	Sigma Aldrich (Cerilliant product)
Hydrocodone	125-29-1	$C_{18}H_{21}NO_3$	299.4	Sigma Aldrich (Cerilliant product)
Ketamine	1867-66-9	$C_{13}H_{16}ClNO$	237.7	Sigma-Aldrich

Lorazepam	846-49-1	$C_{15}H_{10}Cl_2N_2O_2$	321.2	Sigma-Aldrich (Cerilliant product)
Mephedrone	1189726-22-4	$C_{11}H_{15}NO$	177.7	Sigma-Aldrich (Cerilliant product)
Methadone	76-99-3	$C_{21}H_{27}NO$	309.4	Sigma Aldrich (Cerilliant product)
Methamphetamine	4846-07-5	$C_{10}H_{15}N$	149.2	LGC (Cerilliant product)
Morphine	57-27-2	$C_{17}H_{19}NO_3$	285.3	Sigma Aldrich (Cerilliant product)
Morphine-3β-D-glucuronide	20290-09-9	$C_{23}H_{27}NO_9$	461.5	Sigma Aldrich (Cerilliant product)
N-Desmethyltramadol	1018989-94-0	$C_{15}H_{23}NO_2$	249.4	LGC
Nicotine	54-11-5	$C_{10}H_{14}N_2$	162.2	Sigma-Aldrich
Nitrazepam	146-22-5	$C_{15}H_{11}N_3O_3$	281.3	LGC (Cerilliant product)
Norcodeine	467-15-2	$C_{17}H_{19}NO_3$	285.3	Sigma Aldrich (Cerilliant product)
Nordiazepam	1088-11-5	$C_{15}H_{11}ClN_2O$	270.7	Sigma Aldrich (Cerilliant product)
Norephedrine	154-41-6	$C_9H_{13}NO$	151.2	Sigma-Aldrich
Norfluoxetine	107674-50-0	$C_{16}H_{16}F_3NO$	295.3	LGC (Cerilliant product)
Norketamine	79499-59-5	$C_{12}H_{14}ClNO$	223.7	Sigma Aldrich (Cerilliant product)
Normorphine	466-97-7	$C_{16}H_{17}NO_3$	271.3	Sigma Aldrich (Cerilliant product)
Noroxycodone	52446-25--0	$C_{17}H_{19}NO_4$	301.2	Sigma Aldrich (Cerilliant product)
Nortriptyline	894-71-3	$C_{19}H_{21}N$	263.4	Sigma-Aldrich
O-Desmethyltramadol	185453-02-5	$C_{15}H_{23}NO_2$	249.4	LGC
Oxazepam	604-75-1	$C_{15}H_{11}ClN_2O_2$	286.7	Sigma-Aldrich (Cerilliant product)
Oxycodone	76-42-6	$C_{18}H_{21}NO_4$	315.4	Sigma Aldrich (Cerilliant product)
Oxymorphone	76-41-5	$C_{17}H_{19}NO_4$	301.3	Sigma Aldrich (Cerilliant product)
PMA (Para-methoxyamphetamine)	3706-26-1	$C_{10}H_{15}NO$	165.0	LGC
Pseudoephedrine	321-97-1	$C_{10}H_{15}NO$	165.2	Sigma-Aldrich
Sildenafil	139755-83-2	$C_{22}H_{30}N_6O_4S$	474.6	Sigma Aldrich (Cerilliant product)
Temazepam	846-50-4	$C_{16}H_{13}ClN_2O_2$	300.7	Sigma Aldrich (Cerilliant product)
Tramadol	36282-47-0	$C_{16}H_{25}NO_2$	263.4	Sigma-Aldrich
Vardenafil	**224789-1515-5**	$C_{23}H_{32}N_6O_4S$	488.6	Sigma Aldrich (Cerilliant product)
Venlafaxine	99300-78-4	$C_{17}H_{27}NO_2$	277.4	Sigma-Aldrich
Zolpidem	99294-93-6	$C_{19}H_{21}N_3O$	307.4	Sigma Aldrich (Cerilliant product)
Zopiclone	43200-80-2	$C_{17}H_{17}ClN_6O_6$	388.8	LGC

Table S2. Daily wastewater flows

Day	Total daily wastewater flows (M^3 day^{-1})				
	2014	2015	2016	2017	2018
Wednesday	214,229.0	198,950.0	215,806.5	292,715.1	231,919.2
Thursday	208,782.0	197,523.0	307,513.8	284,589.0	227,280.6
Friday	208,644.0	252,682.0	304,422.3	246,701.7	217,694.7
Saturday	204,287.0	220,687.0	294,425.1	223,340.4	225,110.7
Sunday	198,221.0	193,194.0	271,405.8	212,575.5	230,450.4
Monday	199,012.0	197,493.0	249,774.3	208,015.2	218,876.4
Tuesday	216,049.0	204,491.0	235,544.4	211,186.8	219,577.5

Table S3. Daily PNDLs

Compound		Average PNDL (mg day/1000 inh)				
		2014	2015	2016	2017	2018
Benzoylecognine (BEG)	Average	248.2973	306.303	390.4124	754.7391	969.2295
	+/-	21.33318	15.82813	14.82511	21.31492	28.63601
Amphetamine (AMP)	Average	83.06841	82.80847	93.6493	53.75748	121.0263
	+/-	8.268261	4.750731	7.140431	2.148553	16.24645
Methamphetamine (METH)	Average	2.104612	1.256922	0	41.36523	8.673653
	+/-	1.153871	0.875313	0	24.95339	1.134556
3,4-methylenedeoxyamphetamine (MDA)	Average	2.208702	1.947971	0	0.804672	0
	+/-	1.835686	0.466986	0	0.111187	0
3,4-methylenedeoxy methamphetamine (MDMA)	Average	31.46043	53.2289	51.71856	33.04484	46.9303
	+/-	2.010495	2.956396	2.781761	1.058683	0.948299
4-hydroxy-3-methoxy methamphetamine (HMMA)	Average	8.128021	15.44585	0	55.50796	0
	+/-	1.186781	0	0	2.843198	0
Caffeine	Average	38.58803	44.74016	41.99957	52.15982	52.77973
	+/-	1.94694	0.789701	2.378755	1.551367	3.208756
1,7-dimethylxanthine (1,7-DMX)	Average	24.51775	60.14676	83.10163	99.23699	112.7096
	+/-	4.425167	2.006441	6.309632	4.845674	6.683458
Nicotine ((-)-NIC)	Average	1.423007	1.696898	2.625194	5.550752	7.179193
	+/-	0.7798	0.111869	0.527135	2.109184	0.815735

Cotinine (COT)	Average	0.506785	0.487587	0.413297	0.468155	0.54007
	+/-	0.093108	0.021205	0.018833	0.014314	0.022275
Creatinine (CREAT)	Average	0.088253	0.204459	0	0.180037	0.209353
	+/-	0.017113	0.029252	0	0.016912	0.019039
Codeine (COD)	Average	486.0118	460.5703	389.6839	456.3295	570.6245
	+/-	45.01354	30.59654	35.09607	21.8931	19.19197
Norcodeine (NorCOD)	Average	0	0	0	54.57926	0
	+/-	0	0	0	1.065644	0
Noroxycodone (NorOXY)	Average	5.83519	7.44451	0	56.87295	0
	+/-	3.021134	1.772931	0	0.531392	0
Hydrocodone (HYCDNE)	Average	3.525357	7.388551	0	137.968	268.6697
	+/-	5.438296	2.710745	0	23.21353	5.375476
Oxymorphone (OXYMORPH)	Average	4.854138	5.439319	0	45.70448	0
	+/-	2.702657	1.327378	0	0.406306	0
Morphine (MORPH)	Average	145.1919	290.368	260.9531	226.6823	225.8408
	+/-	9.629086	49.29619	9.004827	1.585444	2.498178
Normorphine (NorMORPH)	Average	36.83189	318.7468	0	239.9074	0
	+/-	9.958646	3.413121	0	9.857387	0
Dihydromorphine (DHMORPH)	Average	6.341436	0	0	3.922981	0
	+/-	1.574158	0	0	0.54557	0
Dihydrocodeine (DHCOD)	Average	98.76037	68.70265	45.37795	36.02608	0.056805
	+/-	11.1793	5.156476	6.060355	2.212172	0.00369
Methadone (MDONE)	Average	12.57207	13.79809	8.551855	11.55235	0
	+/-	1.137421	0.896985	0.790081	0.466848	0
Tramadol (TRAM)	Average	324.1231	225.9863	101.0895	228.4439	101.3231
	+/-	13.51263	16.94739	14.01864	13.76417	5.25227
Temazepam (TEMAZE)	Average	65.7856	25.14433	0	68.58208	714.7638
	+/-	25.32444	3.645201	0	8.840072	113.9894

Oxazepam (OXAZE)	Average	24.13408	0	0	111.1557	86.65784
	+/-	25.33208	0	0	5.909581	10.02523
Venlafaxine (VENLA)	Average	45.20403	38.76657	81.1626	130.3215	85.206
	+/-	3.767817	3.665721	3.872556	7.28028	2.238418
Ketamine (KETA)	Average	62.13813	27.91556	102.5845	118.564	205.3144
	+/-	3.561798	1.800038	5.618439	3.207801	4.778964
Vardenafil (VARDEN)	Average	0.224454	0	0	59.25679	0
	+/-	0.288584	0	0	5.39497	0
1S,2R-(-)-Ephedrine (EPH)	Average	5.285351	0	0	0	31.79748
	+/-	1.059189	0	0	0	3.327085
1R,2R-(+)-Pseudoephedrine (PSE)	Average	39.15167	96.4653	109.3234	0	72.70608
	+/-	3.423638	19.22876	12.98509	0	7.374135
Norephedrine (NorEPH)	Average	0	3.416569	0	0	0
	+/-	0	0.924007	0	0	0
Desmethyl venlafaxine (DSMVENLA)	Average	120.658	0	0	337.7837	405.5399
	+/-	5.190345	0	0	33.68885	29.62472
Para-methoxyamphetamine (PMA)	Average	0	0	0	241.2251	0
	+/-	0	0	0	21.0044	0
Cocaine (COC)	Average	115.0255	160.2827	12.98584	445.4593	554.8532
	+/-	6.703992	6.474846	0.892027	11.50117	13.26825
Cocaethylene (CE)	Average	1.085241	7.556583	10.70124	15.22374	25.14734
	+/-	0.099369	0.967827	0.957959	0.526797	0.717654
Anhydroecgonine methyl ester (AEME)	Average	2.064846	3.326668	0	28.97084	0
	+/-	0.370081	0.758871	0	0.614639	0
Heroin (HEROIN)	Average	14.08867	0	0	4.016655	0
	+/-	16.09458	0	0	0	0
6-monoacetly morphine (O-6-MAM)	Average	0.962679	0.855209	0	76.80896	66.12448
	+/-	0.766139	0	0	0.335485	1.824299

Mephedrone (DRONE)	Average	14.73284	25.70685	0	0	0
	+/-	3.028567	6.908809	0	0	0

Table S4. Daily intake

Compound		Average daily intake (mg day/1000 inh)				
		2014	2015	2016	2017	2018
Cocaine (Using BEG PNDL)	Average	578.53	713.69	909.66	1758.54	3489.23
	+/-	49.71	36.88	34.54	49.66	66.72
Amphetamine (Using AMP PNDL)	Average	274.13	273.27	309.04	177.40	338.87
	+/-	27.29	15.68	23.56	7.09	45.49
Methamphetamine (Using METH PNDL)	Average	4.84	2.89	0.00	95.14	20.82
	+/-	2.65	2.01	0.00	57.39	2.72
3,4-methylenedeoxy methamphetamine (Using MDMA PNDL)	Average	138.43	234.21	227.56	145.40	206.49
	+/-	8.85	13.01	12.24	4.66	4.17
Caffeine (Using 1,7-DMX PNDL)	Average	573.72	1407.43	1944.58	2322.15	2637.40
	+/-	103.55	46.95	147.65	113.39	156.39
Nicotine (Using COT PNDL)	Average	1.58	1.52	1.29	1.46	1.69
	+/-	0.29	0.07	0.06	0.04	0.07
Codeine (Using COD PNDL)	Average	1676.74	1588.97	1344.41	1574.34	1968.65
	+/-	155.30	105.56	121.08	75.53	66.21
Dihydrocodeine (Using DHCOD PNDL)	Average	182.71	127.10	83.95	66.65	0.11
	+/-	20.68	9.54	11.21	4.09	0.01
Morphine (Using MORPH PNDL)	Average	187.30	374.57	336.63	292.42	291.33
	+/-	12.42	63.59	11.62	2.05	3.22
Methadone (Using MDONE PNDL)	Average	57.20	62.78	38.91	52.56	0.00
	+/-	5.18	4.08	3.59	2.12	0.00
Tramadol (Using TRAM PNDL)	Average	1079.33	752.53	336.63	760.72	337.41
	+/-	45.00	56.43	46.68	45.83	17.49

Venlafaxine (Using VENLA PNDL)	Average	904.08	775.33	1623.25	2606.43	1704.12
	+/-	75.36	73.31	77.45	145.61	44.77
Ketamine (Using KETA PNDL)	Average	205.06	92.12	338.53	391.26	677.54
	+/-	11.75	5.94	18.54	10.59	15.77
1S,2R-(-)-Ephedrine (Using EPH PNDL)	Average	6.87	0.00	0.00	0.00	41.34
	+/-	1.38	0.00	0.00	0.00	4.33
1R,2R-(+)-Pseudoephedrine (Using PSE PNDL)	Average	43.07	106.11	120.26	0.00	79.98
	+/-	3.77	21.15	14.28	0.00	8.11
Heroin (Using O-6-MAM PNDL)	Average	83.66	74.32	0.00	6674.70	5746.22
	+/-	66.58	0.00	0.00	29.15	158.53
Heroin (Using MORPH AND CODEINE)	Average	143.59	628.07	221.74	90.86	27.87
	+/-	40.81	179.42	53.40	25.70	13.54
Mephedrone (Using DRONE PNDL)	Average	95.76	167.09	0.00	0.00	0.00
	+/-	19.69	44.91	0.00	0.00	0.00

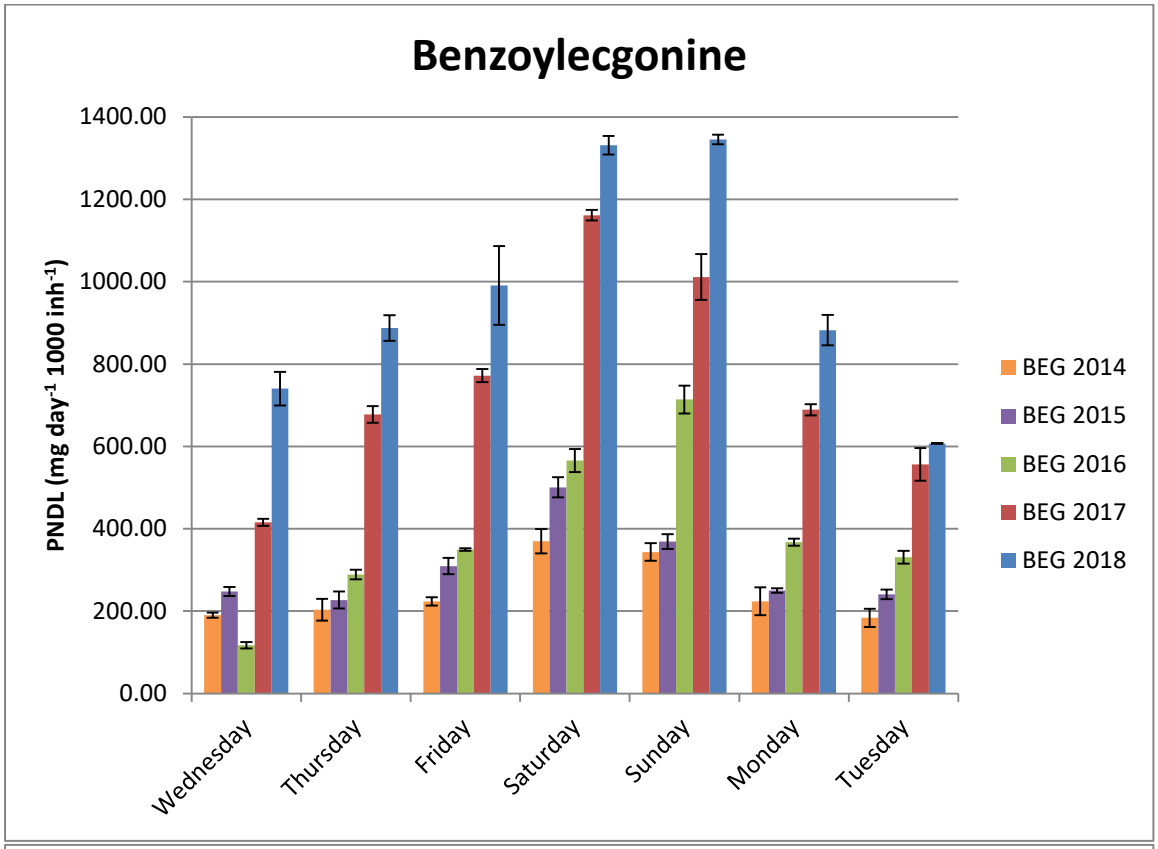
Table S5. Average daily prescription data (mg day^{-1} 1000 inh^{-1})

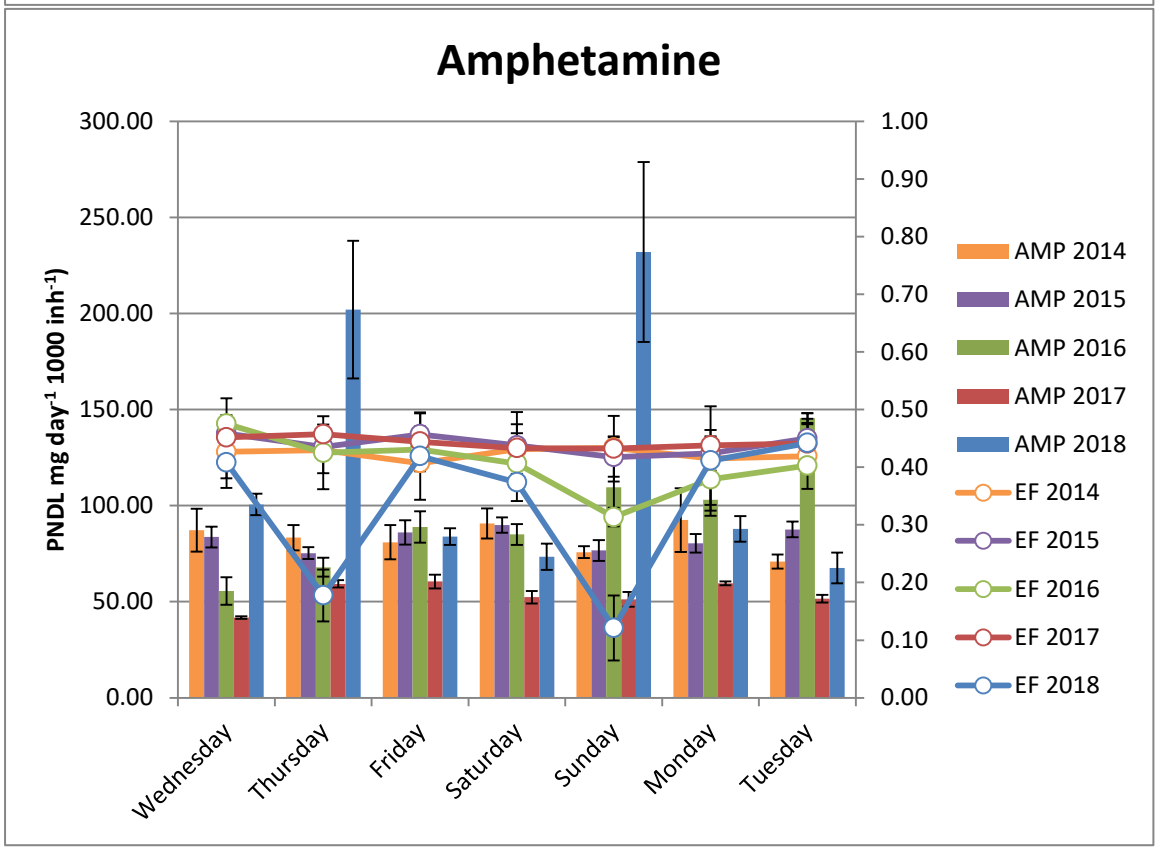
Compound	2014		2015		2016		2017		2018	
	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
Amitriptyline	415.14	16.21	415.83	16.27	423.61	15.99	420.18	13.29	415.65	7.02
Codeine	1798.84	69.98	1810.92	56.17	1800.55	73.95	1764.64	50.82	1721.85	39.34
Diamorphine	1.52	0.24	1.35	0.52	0.67	0.14	0.52	0.07	0.52	0.16
Diazepam	26.93	0.97	25.53	0.91	25.29	0.79	23.91	0.74	22.38	0.60
Dihydrocodeine	289.70	10.00	261.00	11.44	235.12	11.98	207.16	7.92	189.56	5.48
Ephedrine	0.12	0.04	0.13	0.07	0.10	0.01	0.10	0.04	0.11	0.03
Fluoxetine	150.01	6.74	153.43	5.74	154.16	6.48	156.37	6.00	156.22	4.06
Lorazepam	1.39	0.07	1.31	0.08	1.21	0.08	1.10	0.05	1.04	0.04
Methadone	73.31	3.19	76.16	4.01	79.58	3.18	77.52	3.18	77.98	3.22
Morphine	174.78	7.74	178.66	8.21	181.95	6.94	172.64	5.77	161.95	3.84
Nicotine	4.71	0.77	3.35	0.82	2.79	0.41	2.41	0.34	1.99	0.24
Nitrazepam	3.06	0.19	2.52	0.15	2.26	0.09	2.03	0.14	1.76	0.06
Oxazepam	1.23	0.12	1.12	0.15	1.03	0.23	0.85	0.12	0.65	0.09
Oxycodone	35.47	2.12	36.30	1.63	38.75	2.23	40.43	2.41	40.40	1.58
Pseudoephedrine	5.27	0.97	4.23	0.81	3.59	0.63	2.82	0.62	1.73	0.57
Sildenafil	22.92	4.54	38.03	3.69	45.77	2.81	49.86	2.38	52.73	1.61
Temazepam	17.81	1.24	14.69	0.76	12.75	0.64	11.06	0.64	9.81	0.38
Tramadol	1592.57	63.97	1520.30	55.54	1474.94	58.30	1343.57	49.69	1202.16	26.41
Vardenafil	0.64	0.05	0.54	0.05	0.51	0.06	0.47	0.03	0.39	0.05
Venlafaxine	145.63	11.63	229.27	62.54	339.75	18.18	381.26	20.38	397.82	13.58
Zolpidem	2.86	0.14	2.71	0.13	2.75	0.17	2.67	0.14	2.47	0.12
Zopiclone	25.08	0.88	23.70	1.17	22.58	0.87	21.38	0.55	20.21	0.61

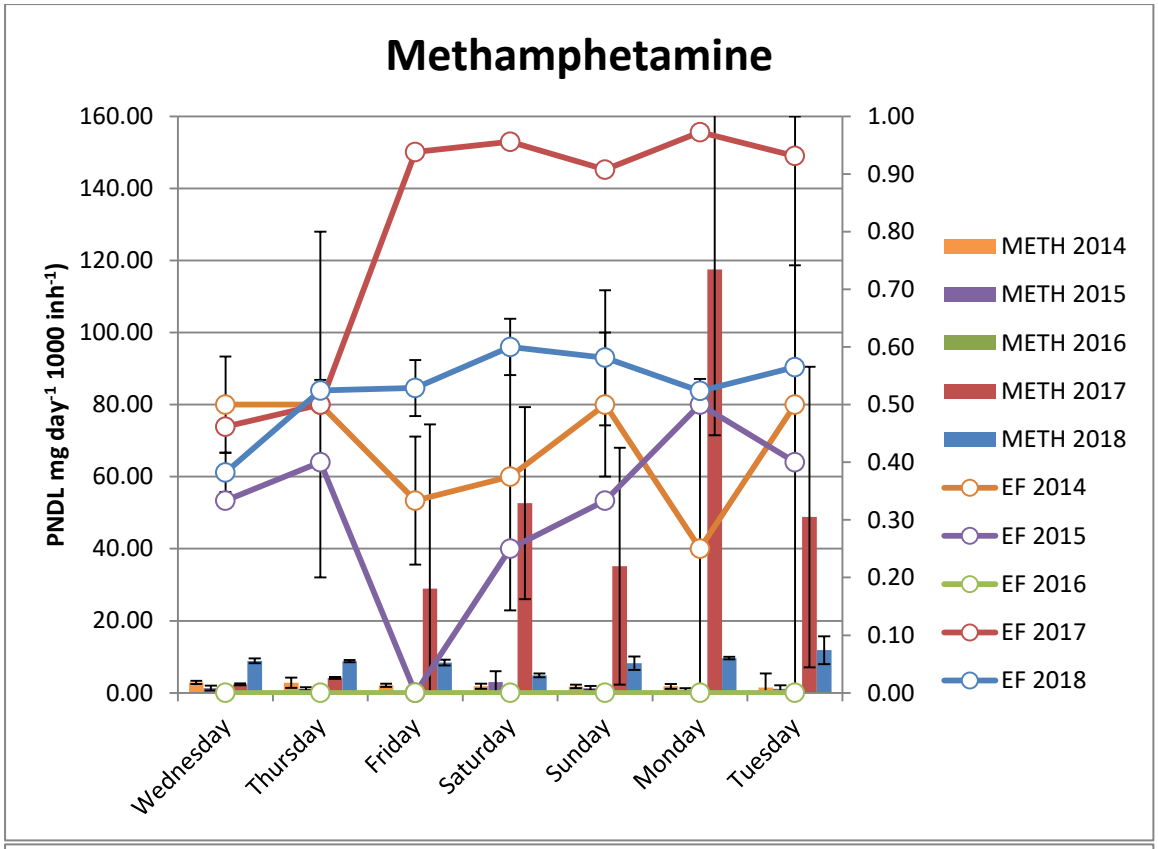
Table S6. Average weekend and weekday PNDL (mg day^{-1} 1000 inh^{-1})

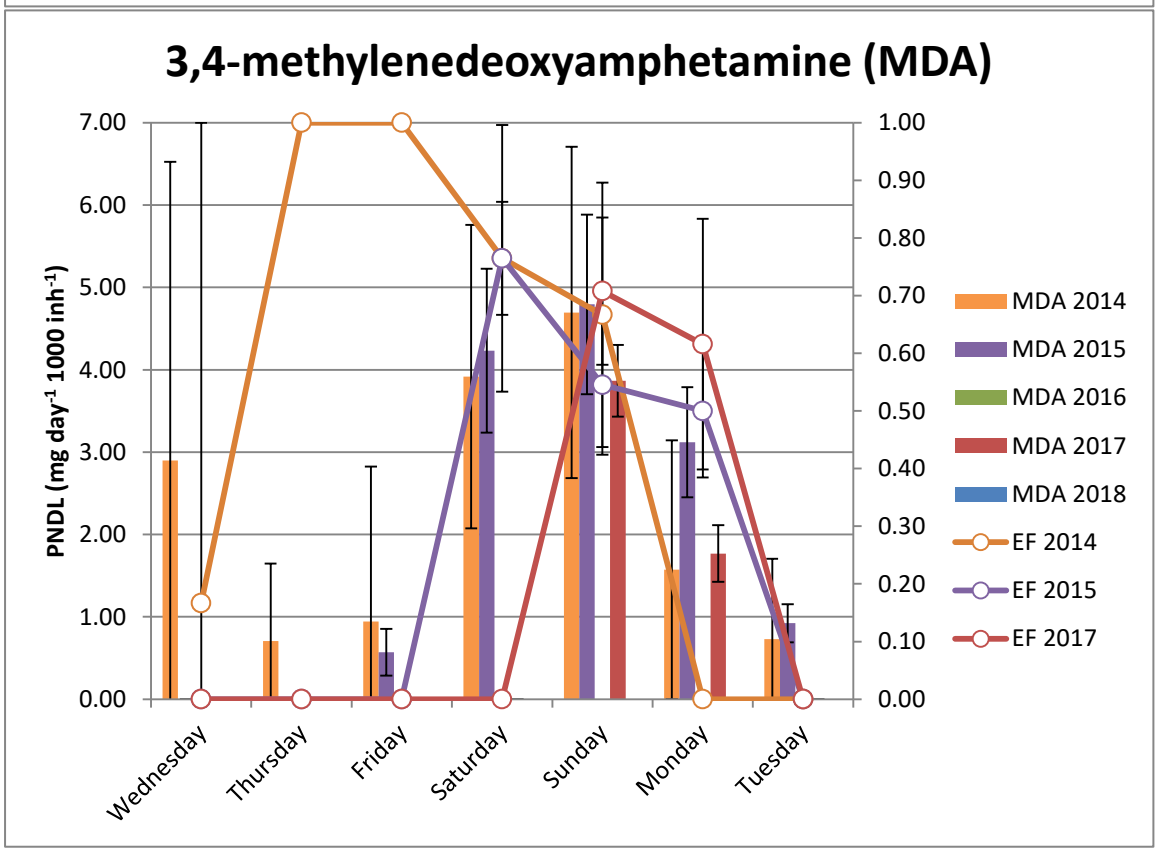
Year	Day	Cocaine PNDL		MDMA PNDL		Ketamine PNDL	
		Average	SD	Average	SD	Average	SD
2014	Weekday	192.52	18.36	17.27	4.43	61.58	5.91
	Weekend	290.13	70.21	85.00	10.90	62.56	3.84
2015	Weekday	238.43	14.95	21.66	4.10	25.69	2.65
	Weekend	358.04	95.54	76.91	34.62	29.53	4.37
2016	Weekday	254.62	93.29	15.39	8.56	89.14	39.48
	Weekend	491.12	150.24	75.91	53.46	114.61	23.79
2017	Weekday	549.85	109.80	13.70	1.60	112.69	14.11
	Weekend	908.40	190.53	47.55	22.59	122.97	20.80
2018	Weekday	744.93	118.18	16.90	1.26	174.50	20.65
	Weekend	1137.45	209.54	69.93	34.23	228.43	6.15

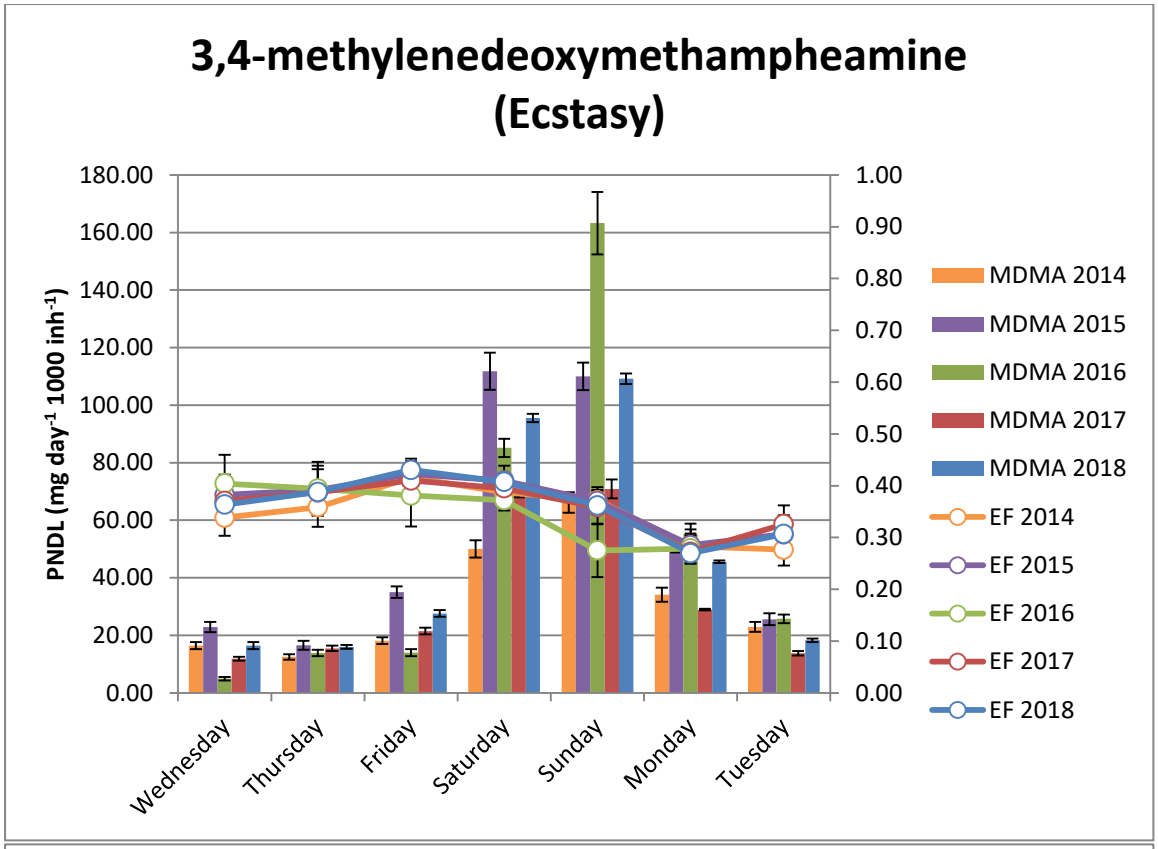
Figure S1. Daily PNDLs

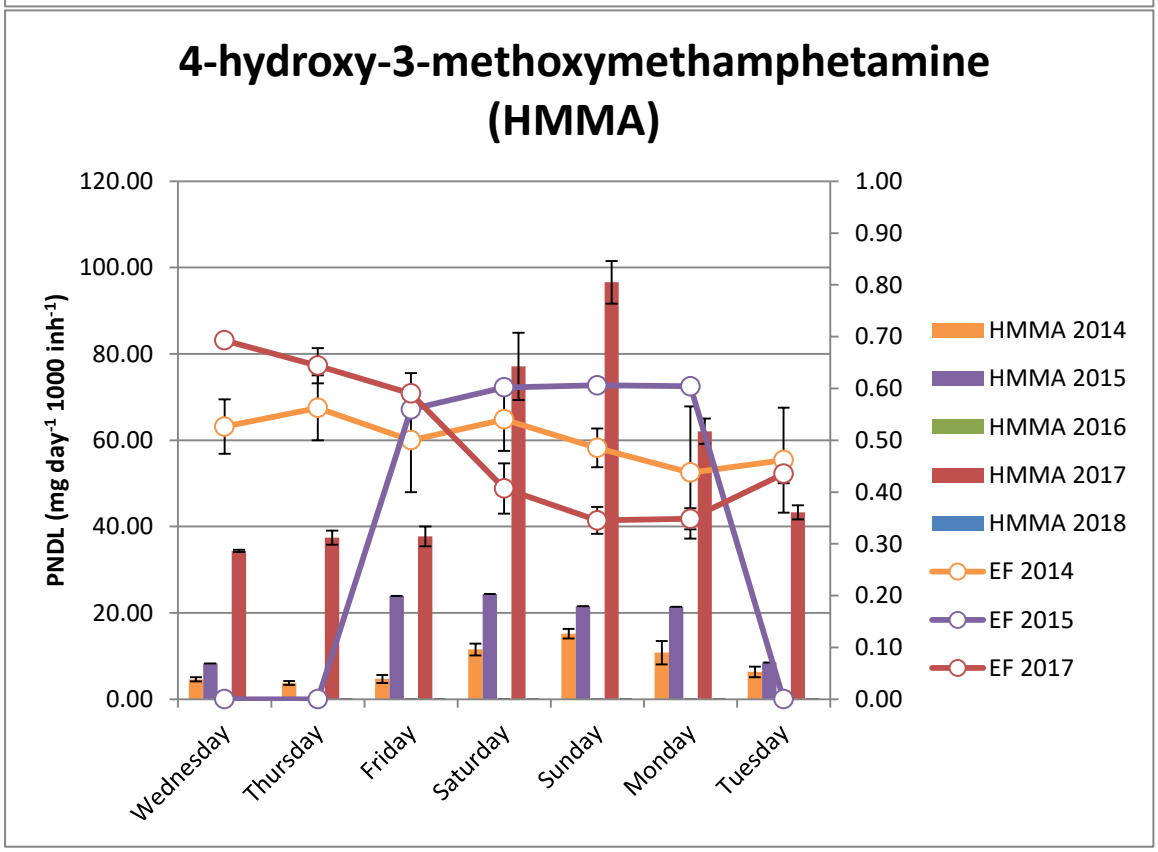


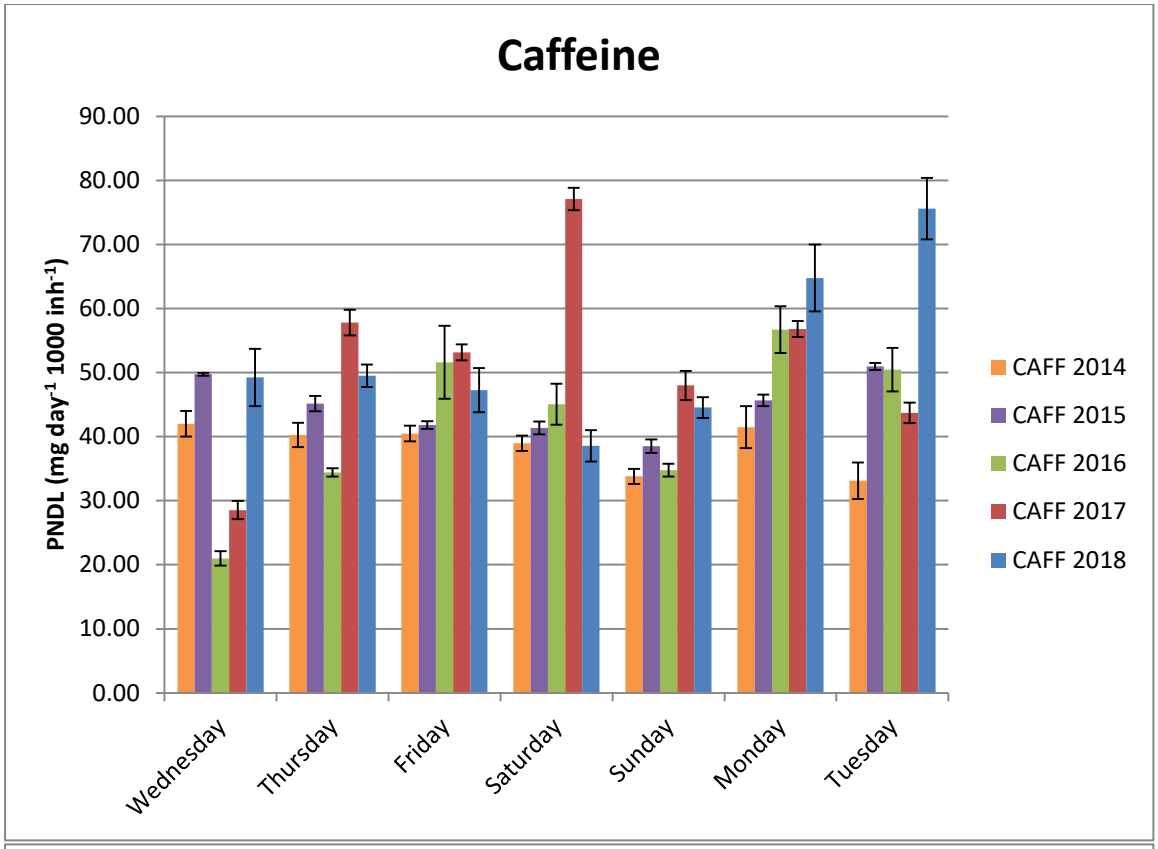


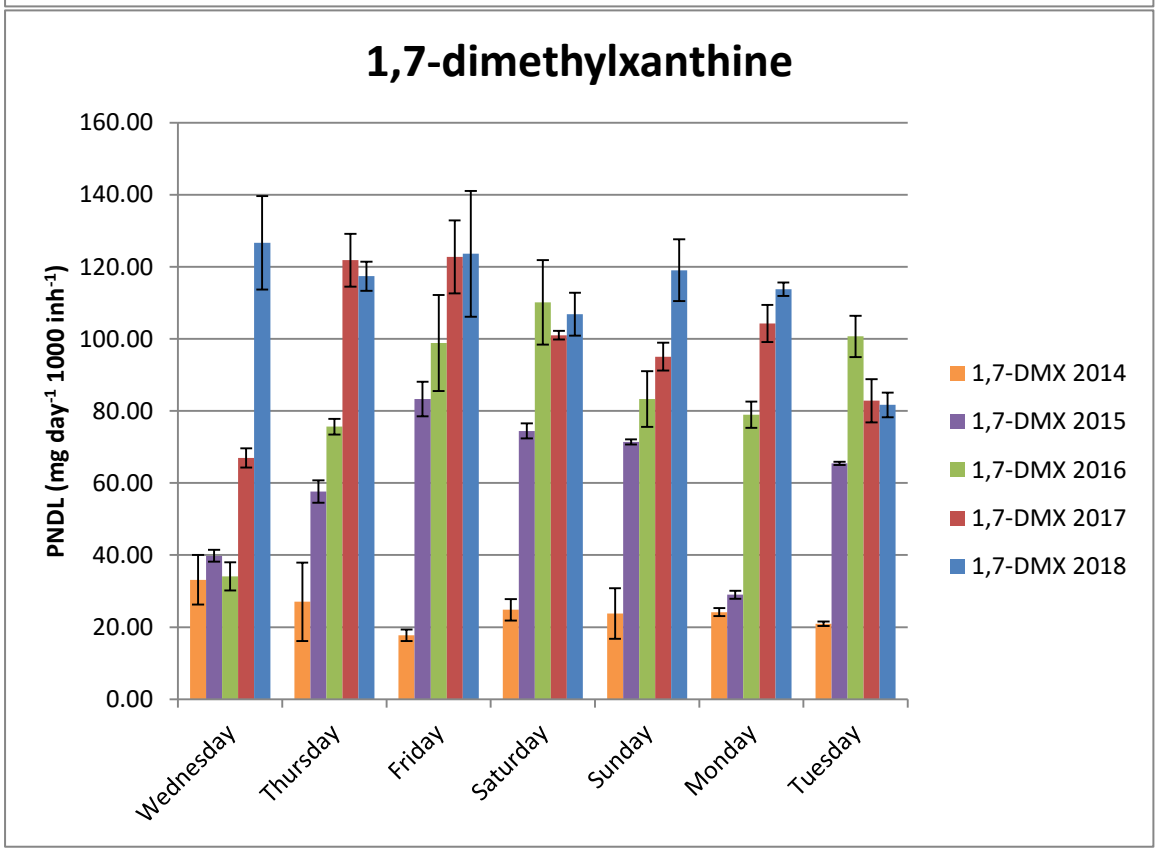

Methamphetamine
PNDL mg day-1 1000 inh-1)
160.00
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
METH 2014
METH 2015
METH 2016
METH 2017
METH 2018
EF 2014
EF 2015
EF 2016
EF 2017
EF 2018

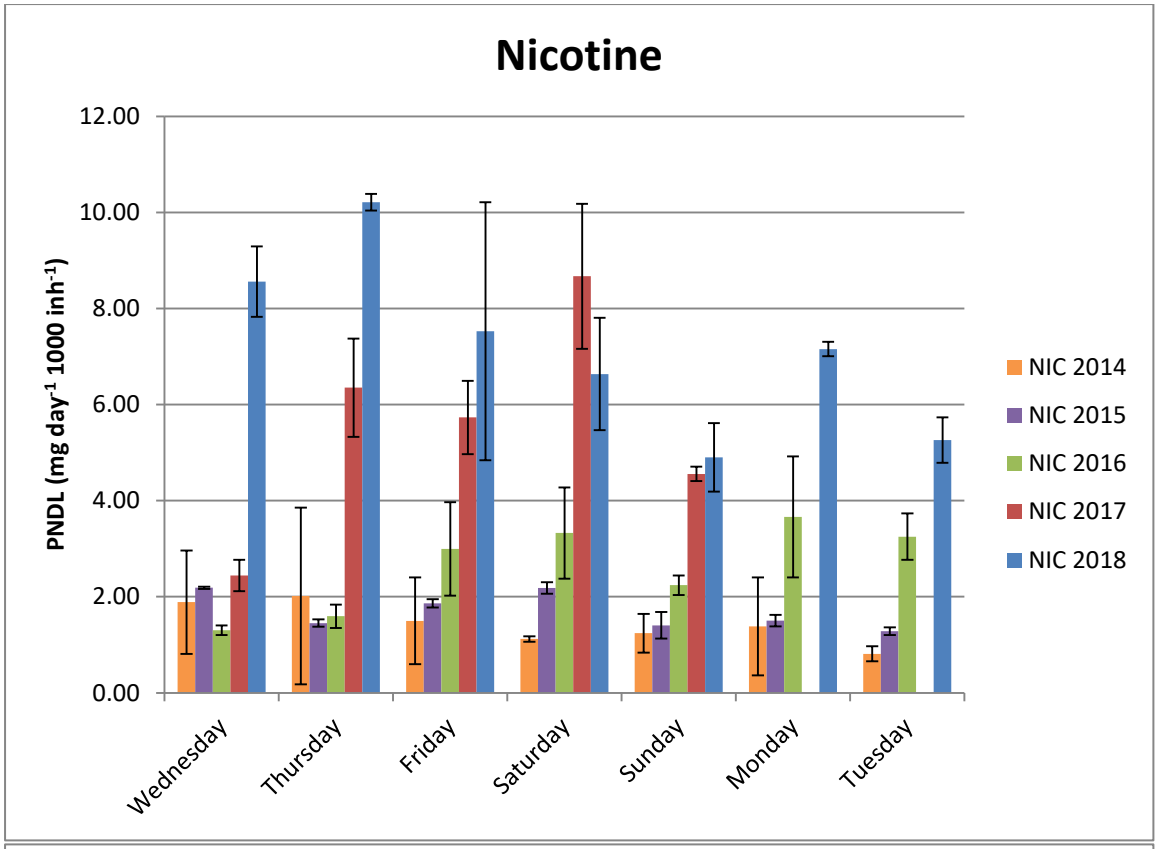

3,4-methylenedeoxyamphetamine (MDA)
PNDL (mg day-1 1000 inh-1)
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
MDA 2014
MDA 2015
MDA 2016
MDA 2017
MDA 2018
EF 2014
EF 2015
EF 2017

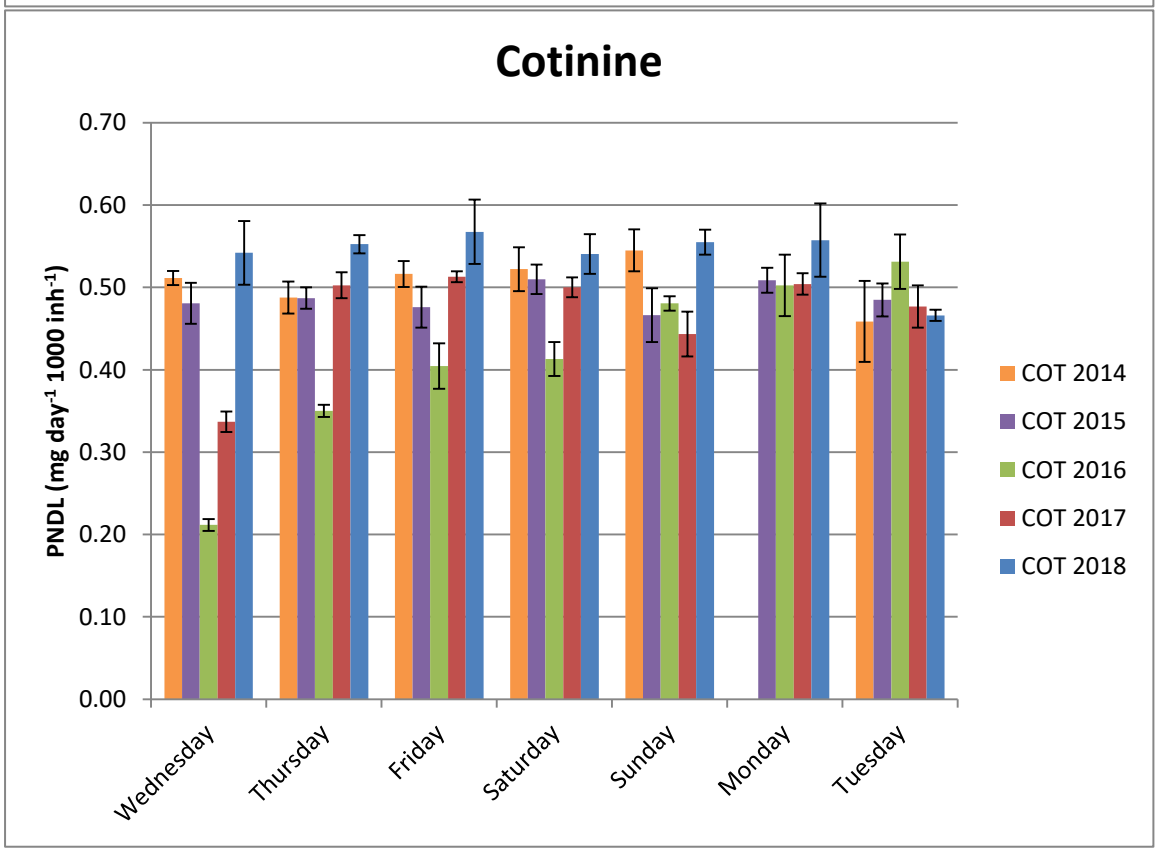

3,4-methylenedeoxymethampheamine (Ecstasy)
PNDL (mg day-1 1000 inh-1)
180.00
160.00
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
MDMA 2014
MDMA 2015
MDMA 2016
MDMA 2017
MDMA 2018
EF 2014
EF 2015
EF 2016
EF 2017
EF 2018

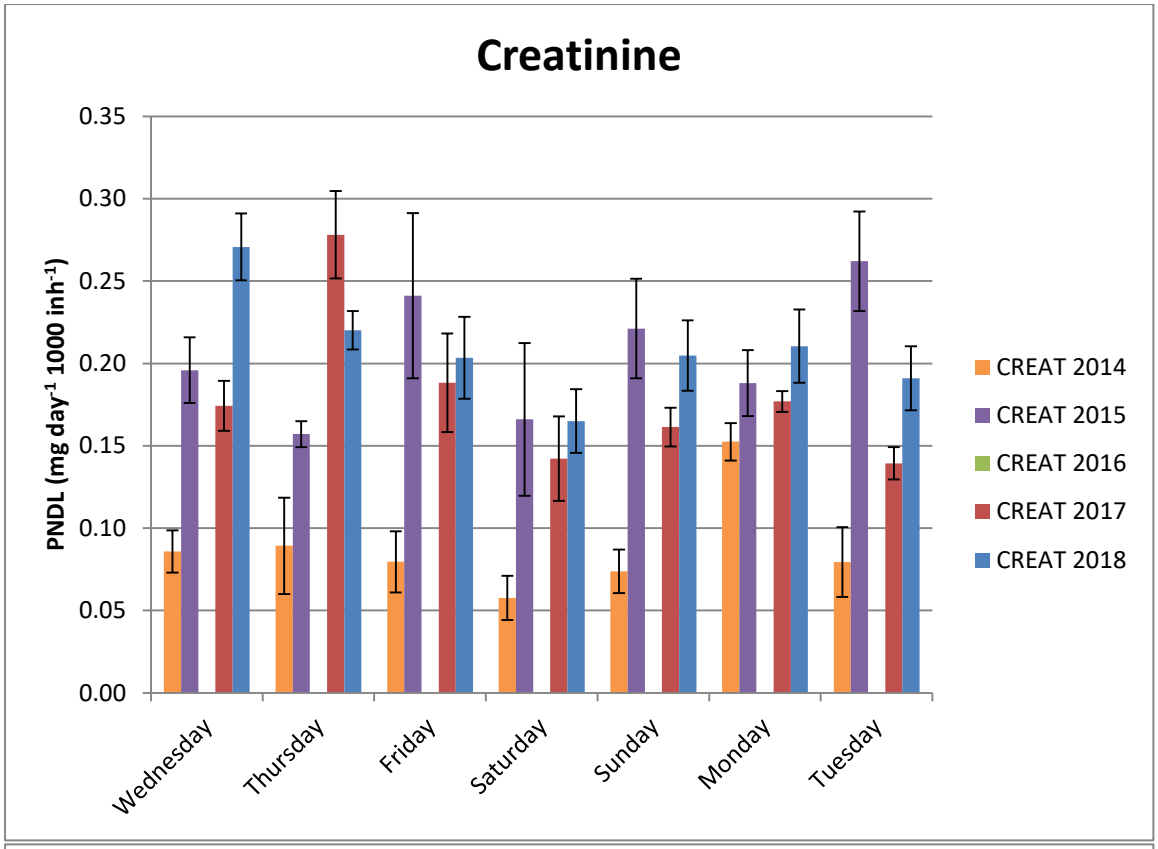

4-hydroxy-3-methoxymethamphetamine (HMMA)
PNDL (mg day-1 1000 inh-1)
120.00
100.00
80.00
60.00
40.00
20.00
0.00
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
HMMA 2014
HMMA 2015
HMMA 2016
HMMA 2017
HMMA 2018
EF 2014
EF 2015
EF 2017

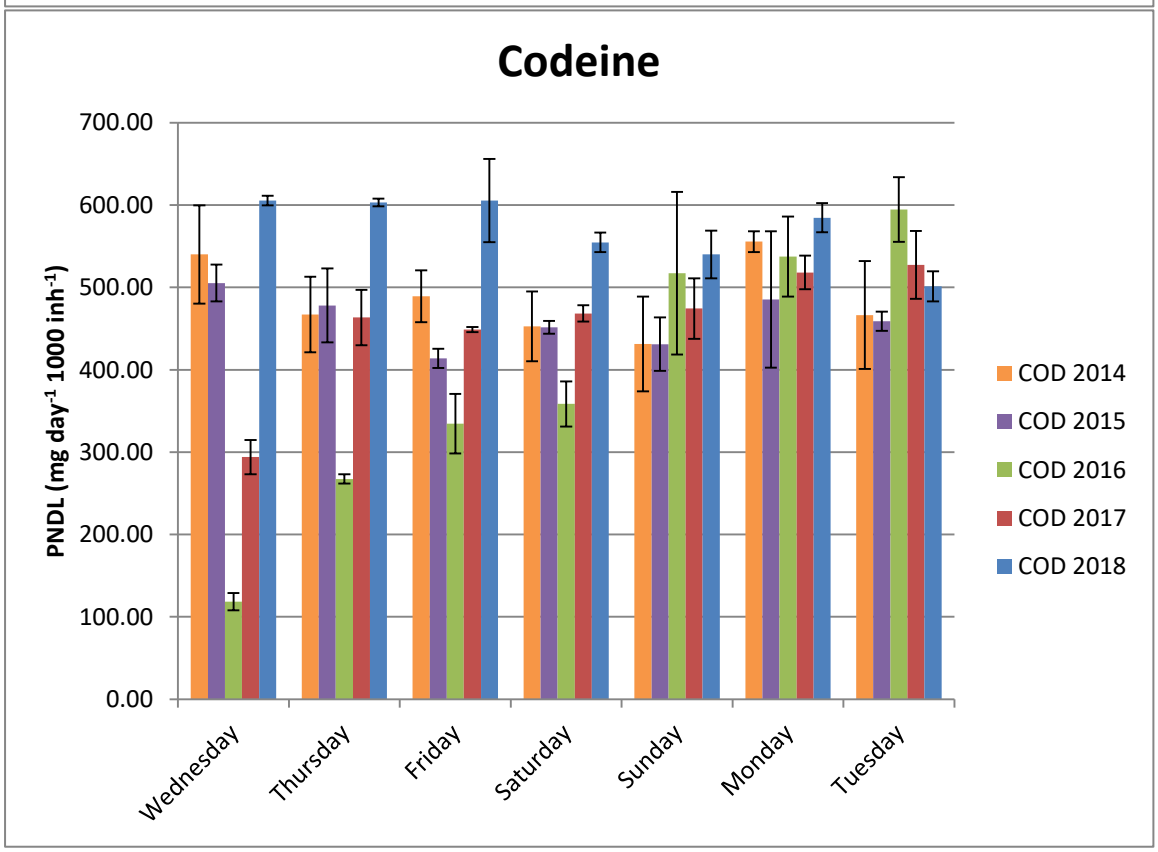

Caffeine
PNDL (mg day-1 1000 inh-1)
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
CAFF 2014
CAFF 2015
CAFF 2016
CAFF 2017
CAFF 2018

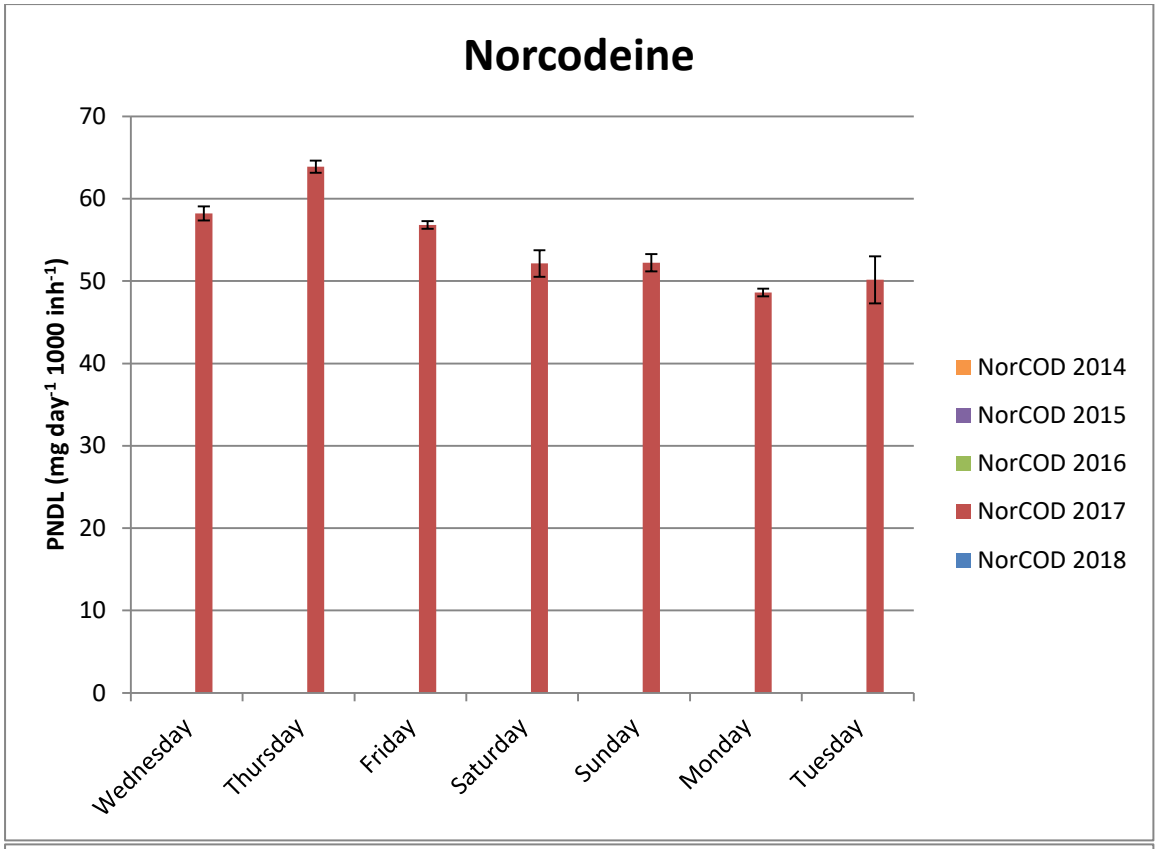

1,7-dimethylxanthine
PNDL (mg day-1 1000 inh-1)
160.00
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
1,7-DMX 2014
1,7-DMX 2015
1,7-DMX 2016
1,7-DMX 2017
1,7-DMX 2018

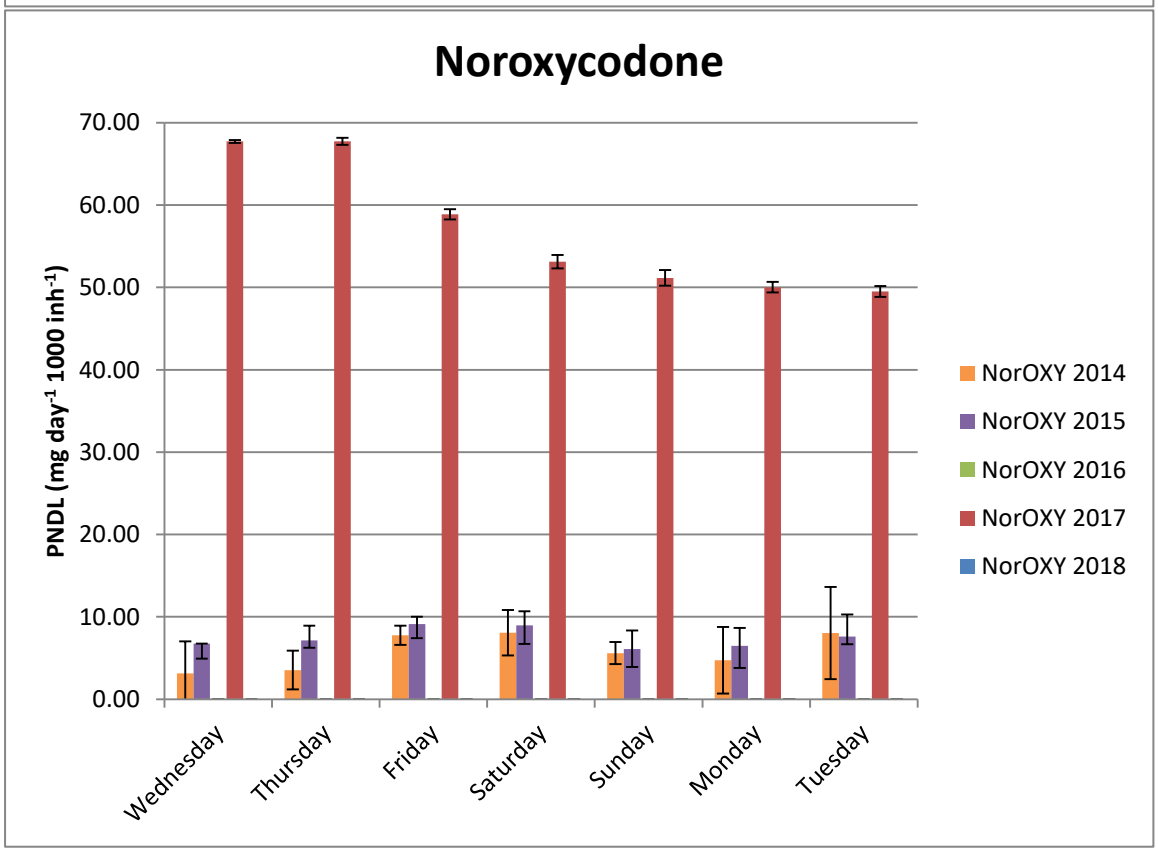

Nicotine
PNDL (mg day-1 1000 inh-1)
12.00
10.00
8.00
6.00
4.00
2.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
NIC 2014
NIC 2015
NIC 2016
NIC 2017
NIC 2018

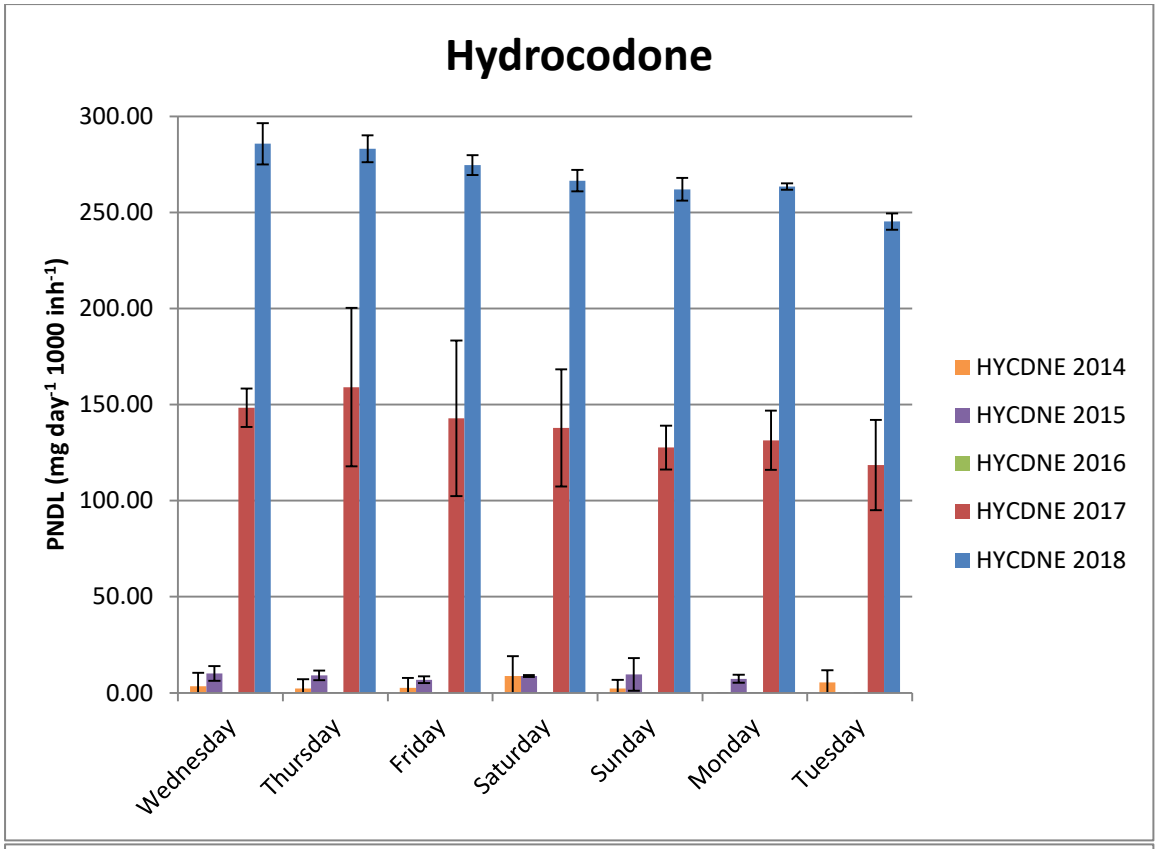

Cotinine
PNDL (mg day-1 1000 inh-1)
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
COT 2014
COT 2015
COT 2016
COT 2017
COT 2018

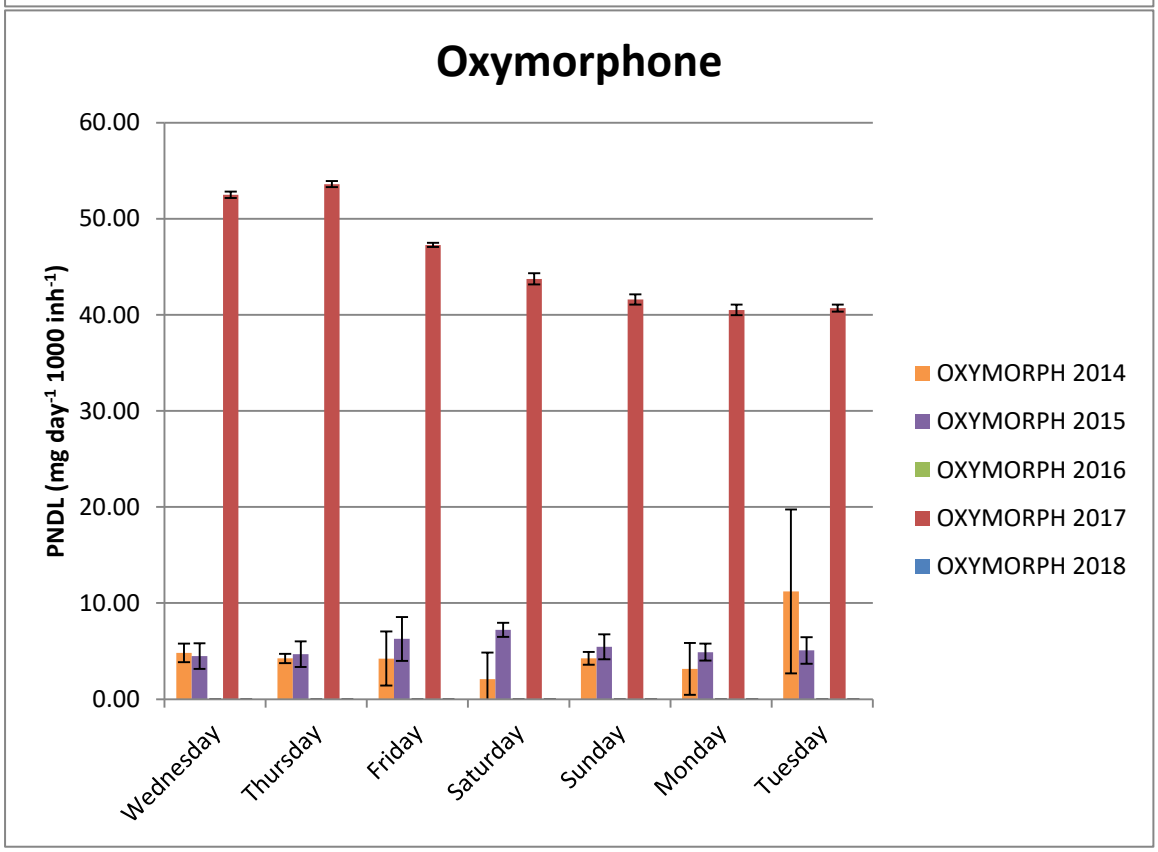

Creatinine
PNDL (mg day-1 1000 inh-1)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
CREAT 2014
CREAT 2015
CREAT 2016
CREAT 2017
CREAT 2018

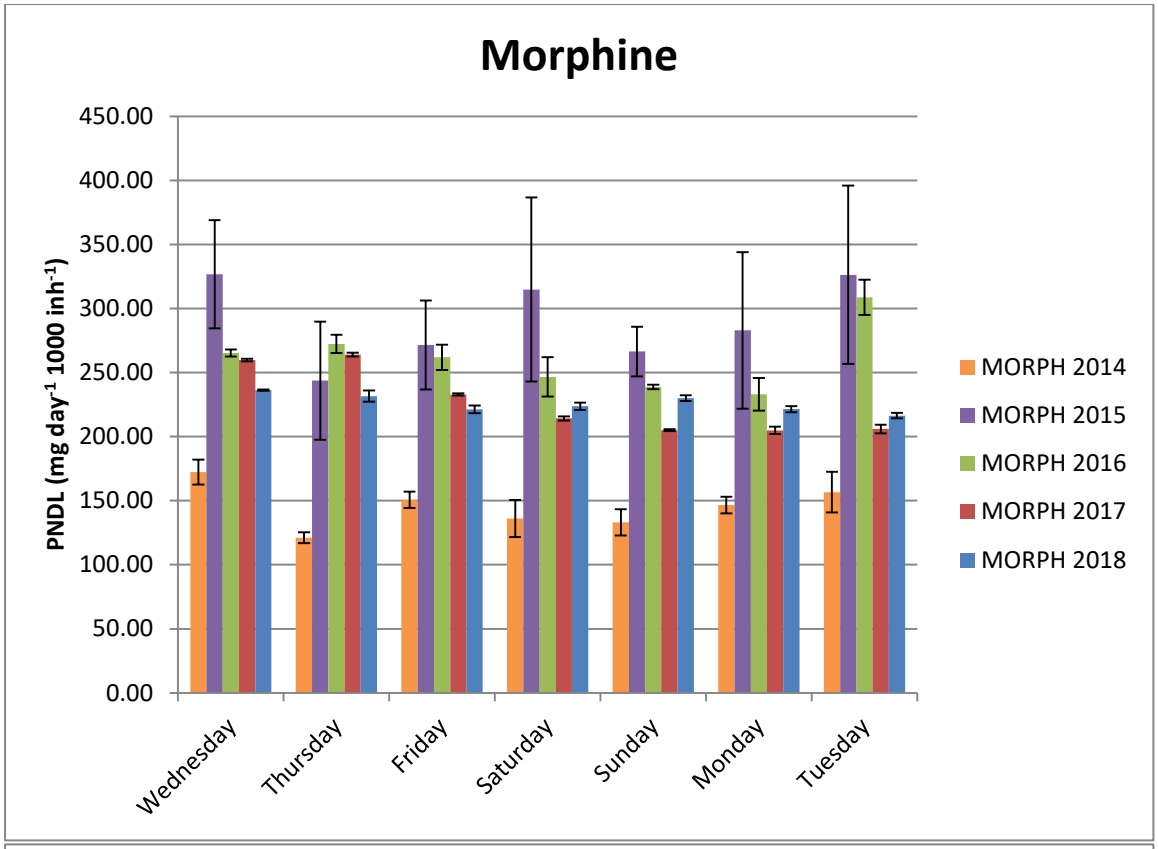

Codeine
PNDL (mg day-1 1000 inh-1)
700.00
600.00
500.00
400.00
300.00
200.00
100.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
COD 2014
COD 2015
COD 2016
COD 2017
COD 2018

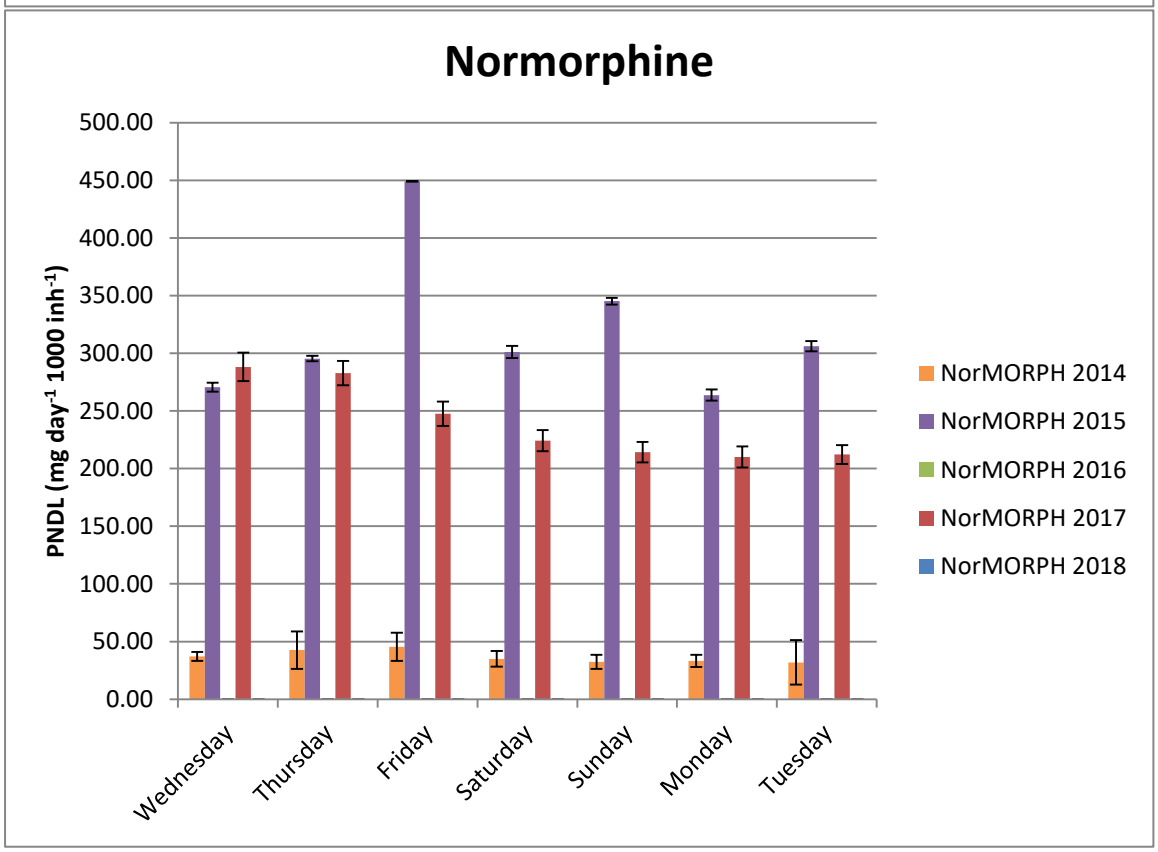

Norcodeine
PNDL (mg day-1 1000 inh-1)
70
60
50
40
30
20
10
0
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
NorCOD 2014
NorCOD 2015
NorCOD 2016
NorCOD 2017
NorCOD 2018

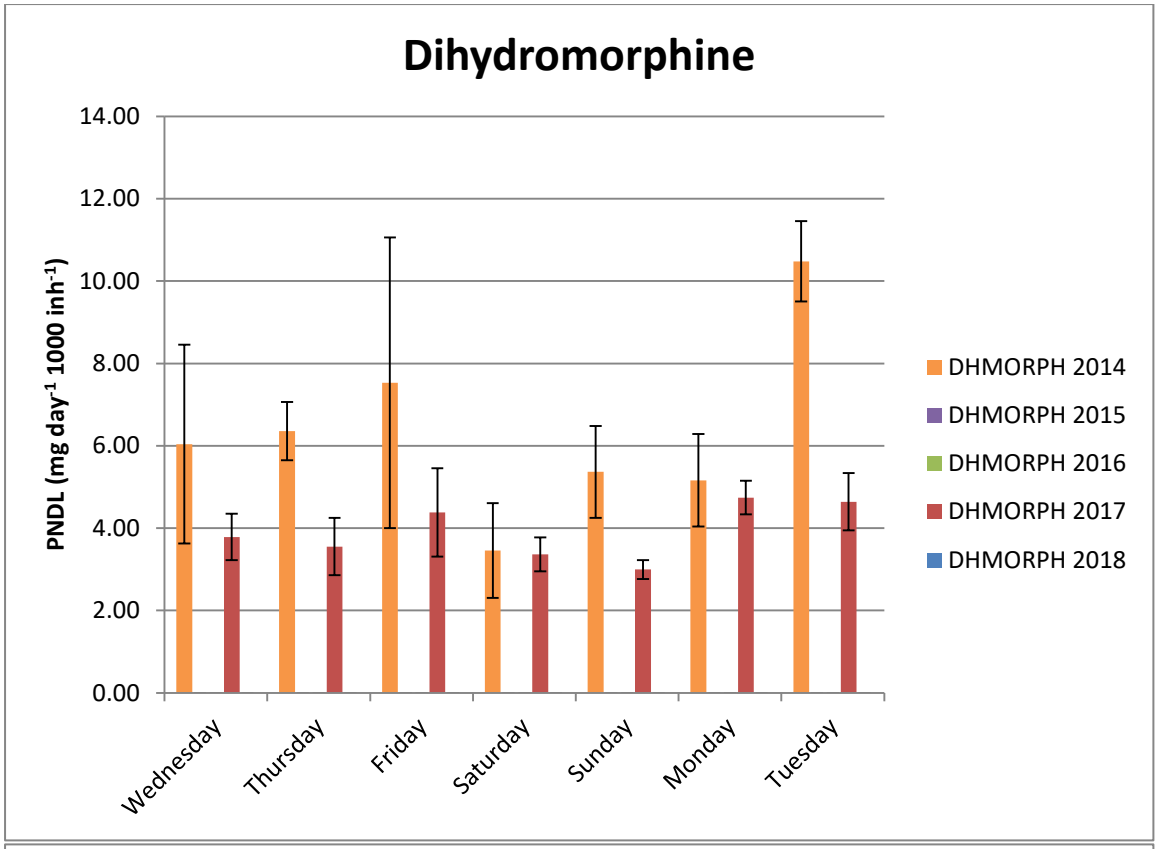

Noroxycodone
PNDL (mg day-1 1000 inh-1)
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
NorOXY 2014
NorOXY 2015
NorOXY 2016
NorOXY 2017
NorOXY 2018

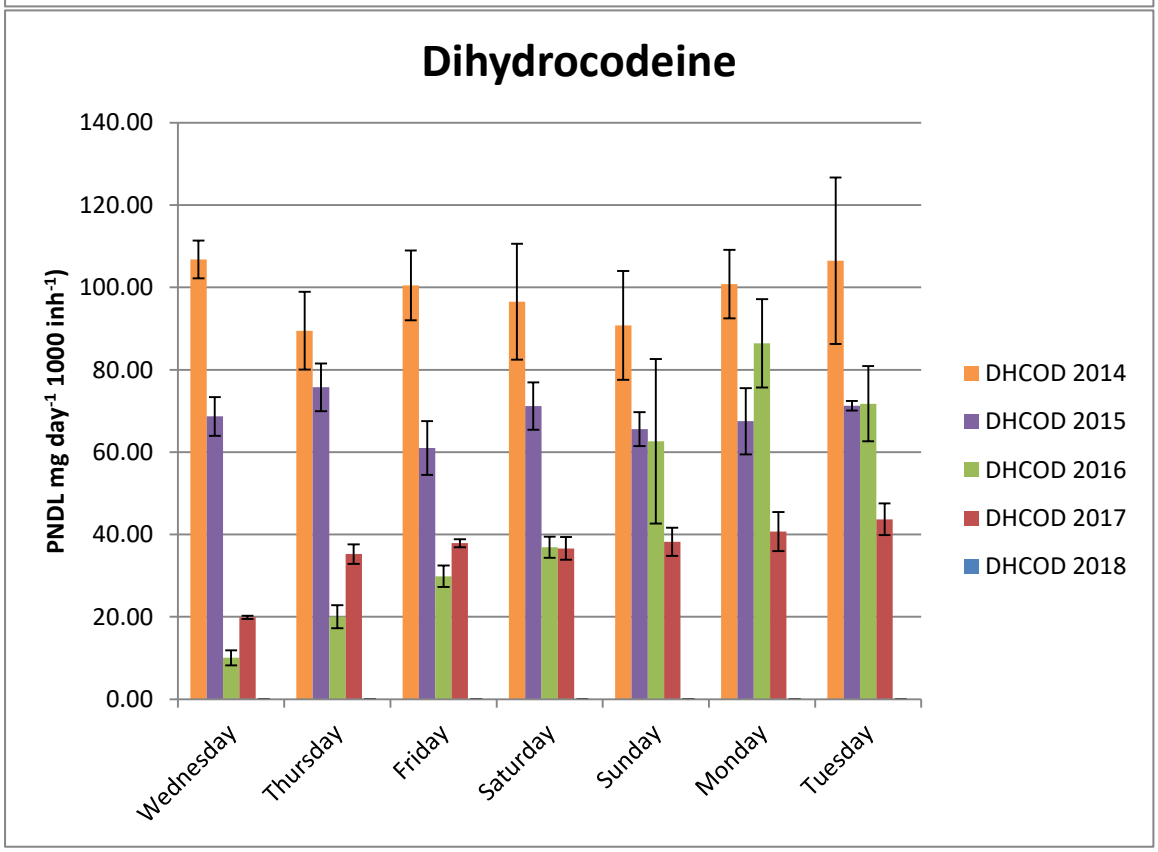

Hydrocodone
PNDL (mg day-1 1000 inh-1)
300.00
250.00
200.00
150.00
100.00
50.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
HYCDNE 2014
HYCDNE 2015
HYCDNE 2016
HYCDNE 2017
HYCDNE 2018

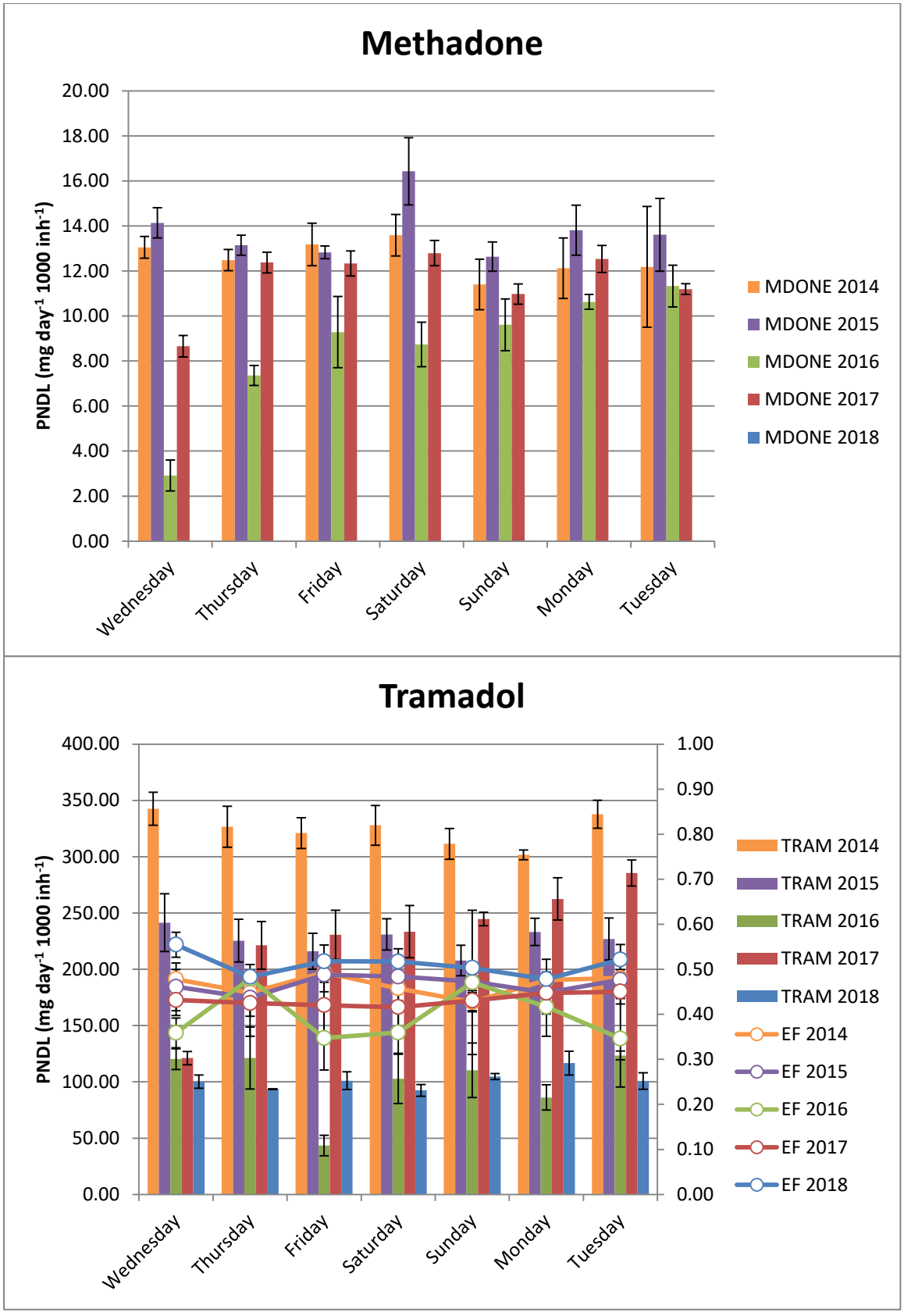

Oxymorphone
PNDL (mg day-1 1000 inh-1)
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
OXYMORPH 2014
OXYMORPH 2015
OXYMORPH 2016
OXYMORPH 2017
OXYMORPH 2018

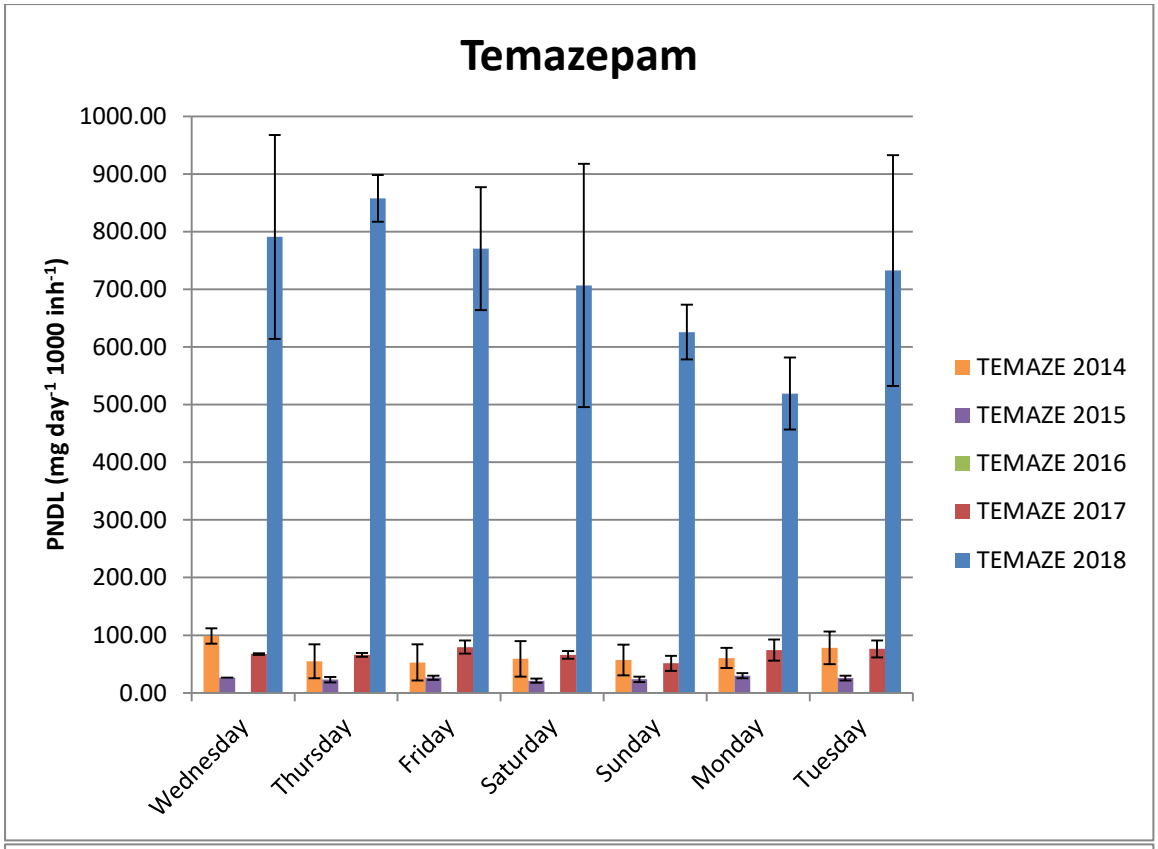

Morphine
PNDL (mg day-1 1000 inh-1)
450.00
400.00
350.00
300.00
250.00
200.00
150.00
100.00
50.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
MORPH 2014
MORPH 2015
MORPH 2016
MORPH 2017
MORPH 2018

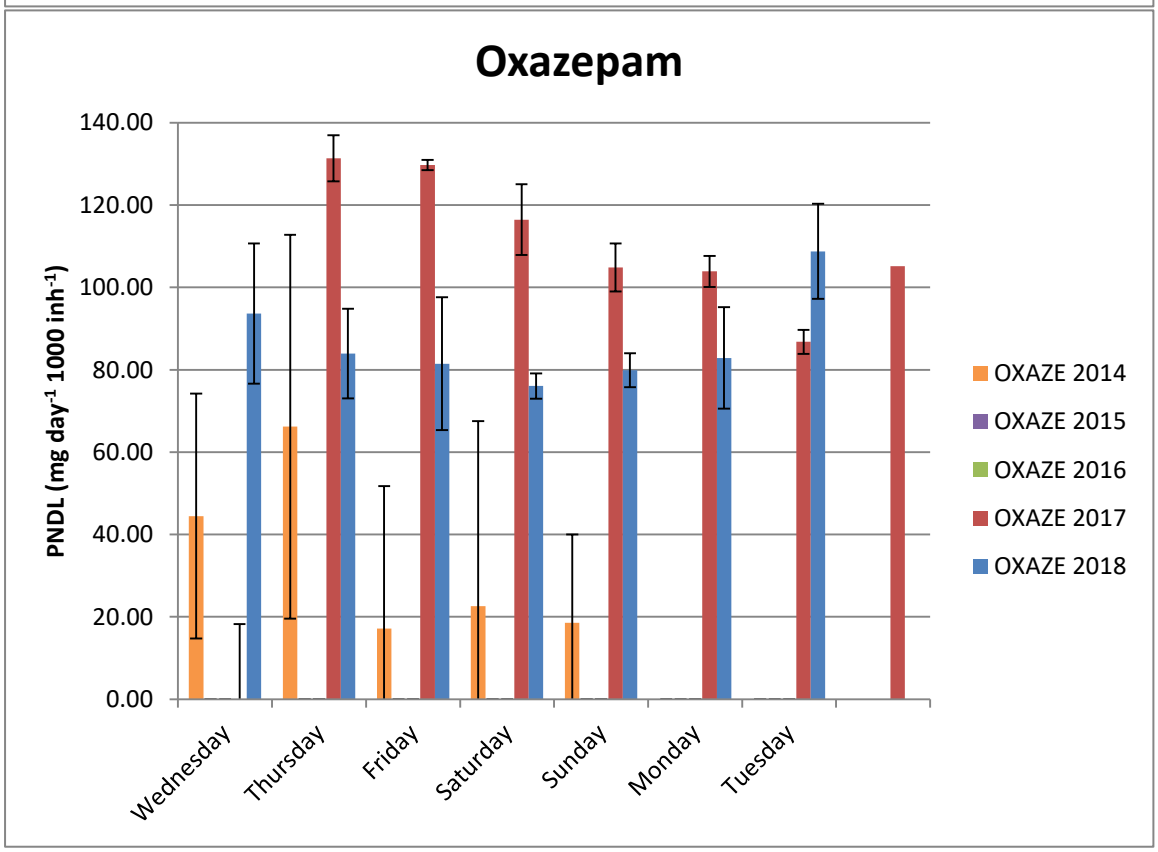

Normorphine
PNDL (mg day-1 1000 inh-1)
500.00
450.00
400.00
350.00
300.00
250.00
200.00
150.00
100.00
50.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
NorMORPH 2014
NorMORPH 2015
NorMORPH 2016
NorMORPH 2017
NorMORPH 2018

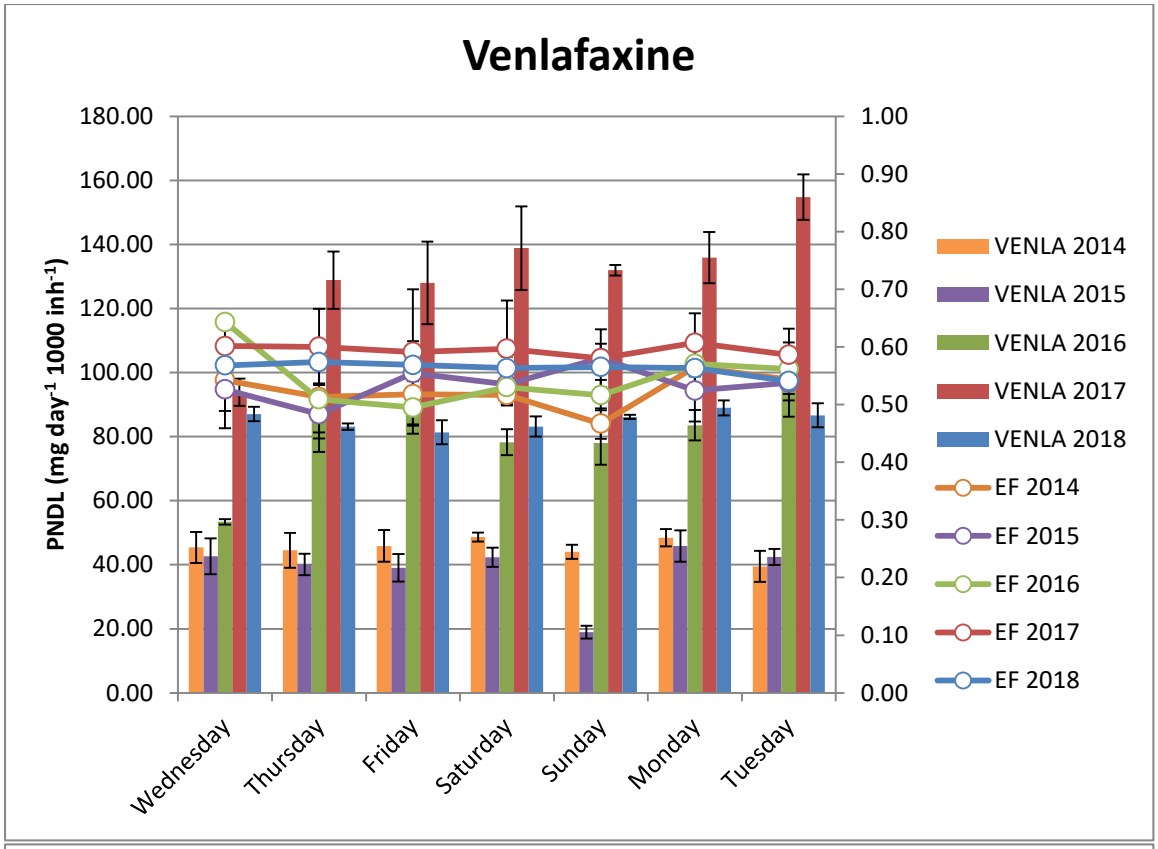

Dihydromorphine
PNDL (mg day-1 1000 inh-1)
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
DHMORPH 2014
DHMORPH 2015
DHMORPH 2016
DHMORPH 2017
DHMORPH 2018

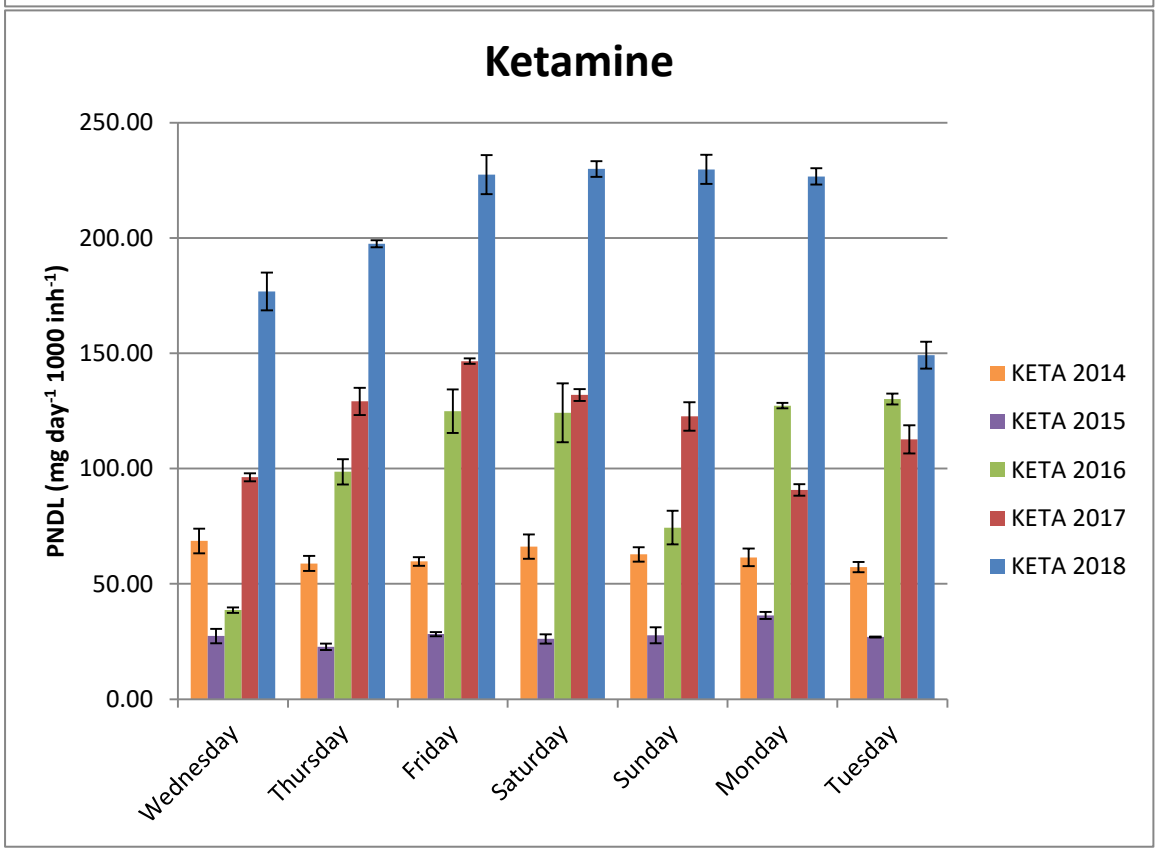

Dihydrocodeine
PNDL mg day-1 1000 inh-1)
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
DHCOD 2014
DHCOD 2015
DHCOD 2016
DHCOD 2017
DHCOD 2018

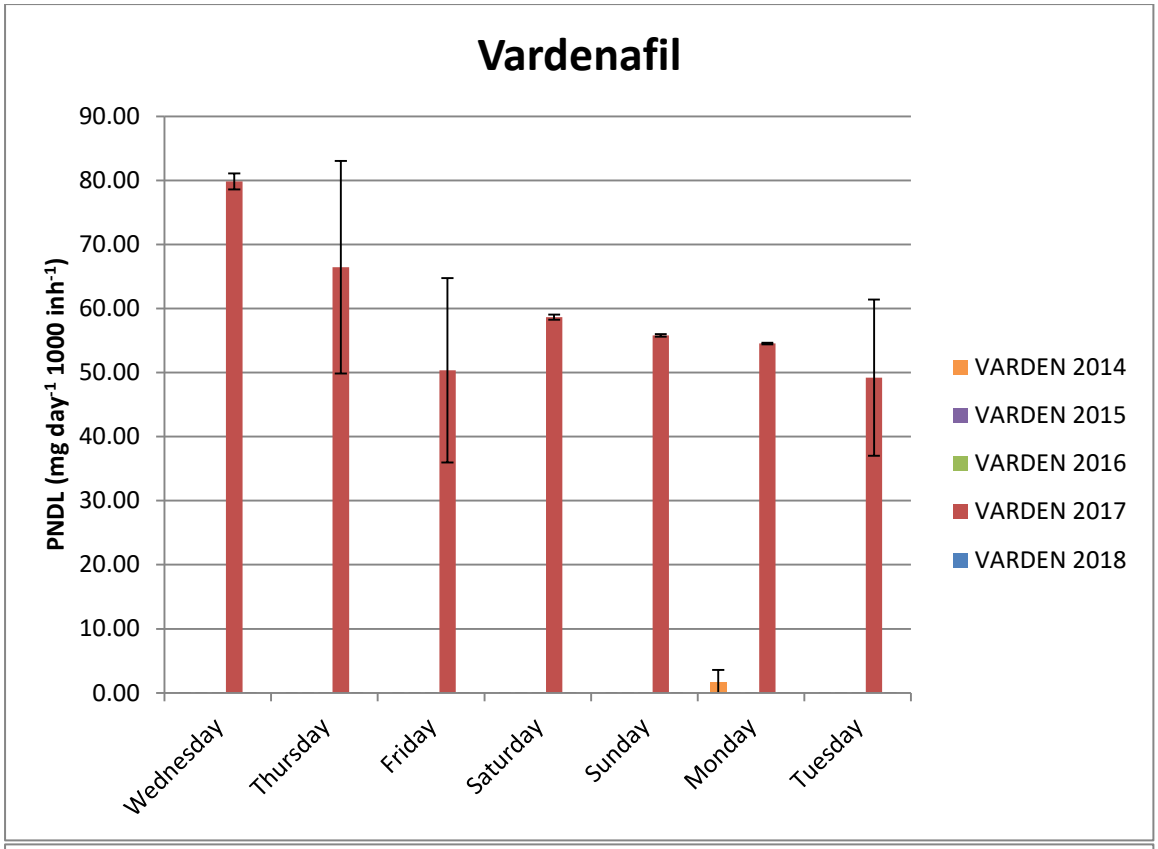

Methadone
PNDL (mg day-1 1000 inh-1)
20.00
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
MDONE 2014
MDONE 2015
MDONE 2016
MDONE 2017
MDONE 2018
Tramadol
PNDL (mg day-1 1000 inh-1)
400.00
350.00
300.00
250.00
200.00
150.00
100.00
50.00
0.00
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
TRAM 2014
TRAM 2015
TRAM 2016
TRAM 2017
TRAM 2018
EF 2014
EF 2015
EF 2016
EF 2017
EF 2018

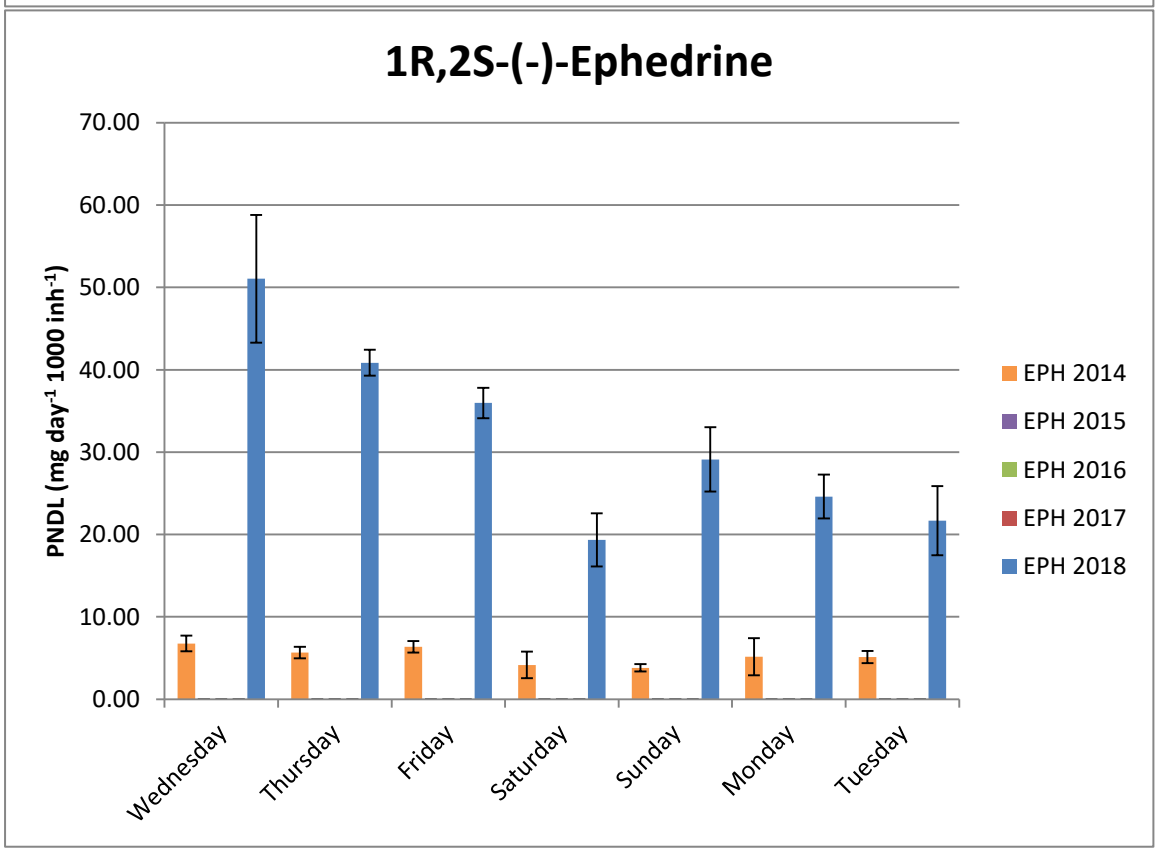

Temazepam
PNDL (mg day-1 1000 inh-1)
1000.00
900.00
800.00
700.00
600.00
500.00
400.00
300.00
200.00
100.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
TEMAZE 2014
TEMAZE 2015
TEMAZE 2016
TEMAZE 2017
TEMAZE 2018

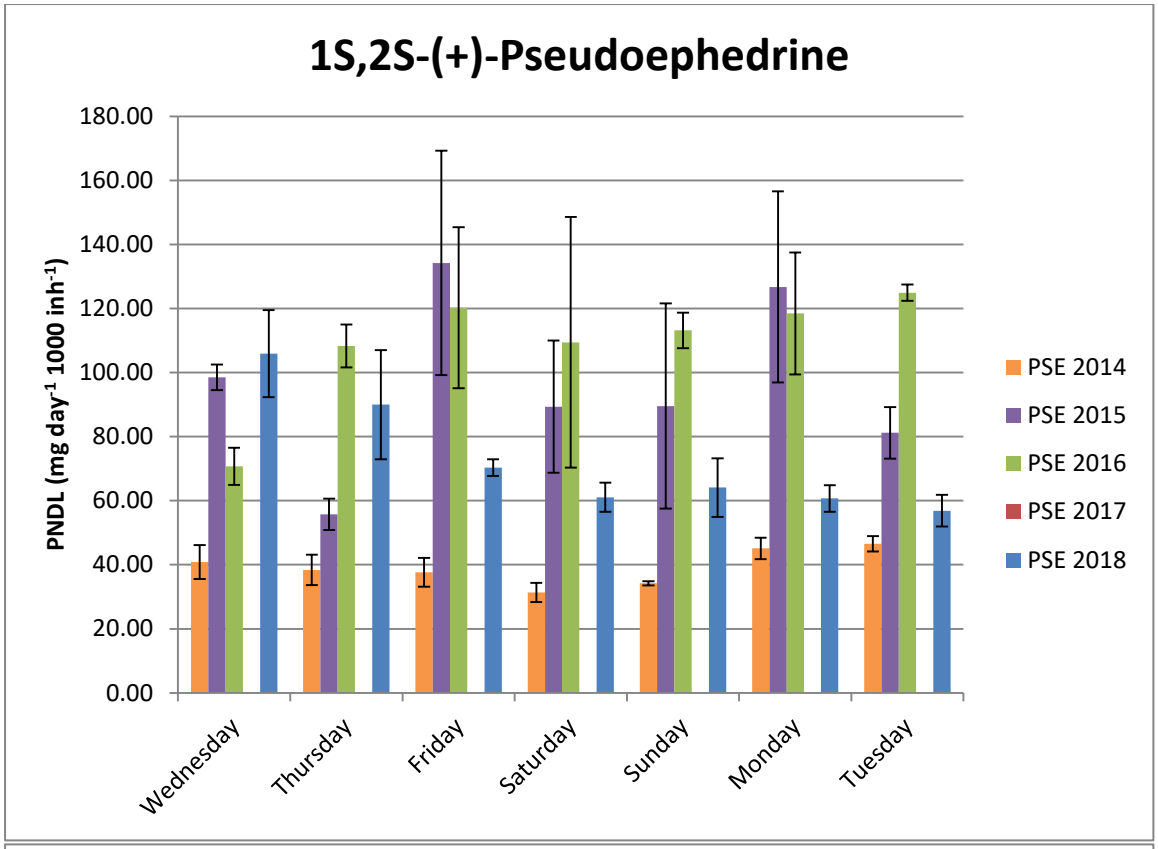

Oxazepam
PNDL (mg day-1 1000 inh-1)
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
OXAZE 2014
OXAZE 2015
OXAZE 2016
OXAZE 2017
OXAZE 2018

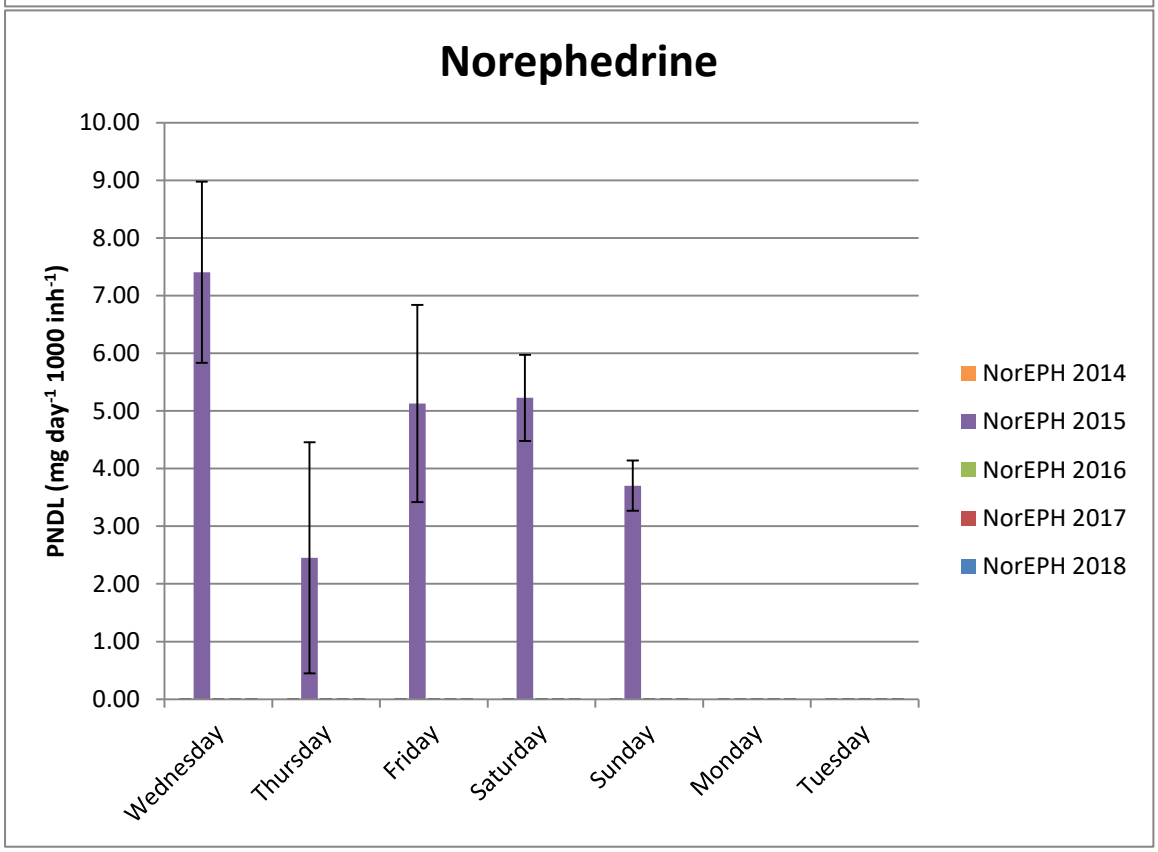

Venlafaxine
PNDL (mg day-1 1000 inh-1)
180.00
160.00
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
VENLA 2014
VENLA 2015
VENLA 2016
VENLA 2017
VENLA 2018
EF 2014
EF 2015
EF 2016
EF 2017
EF 2018

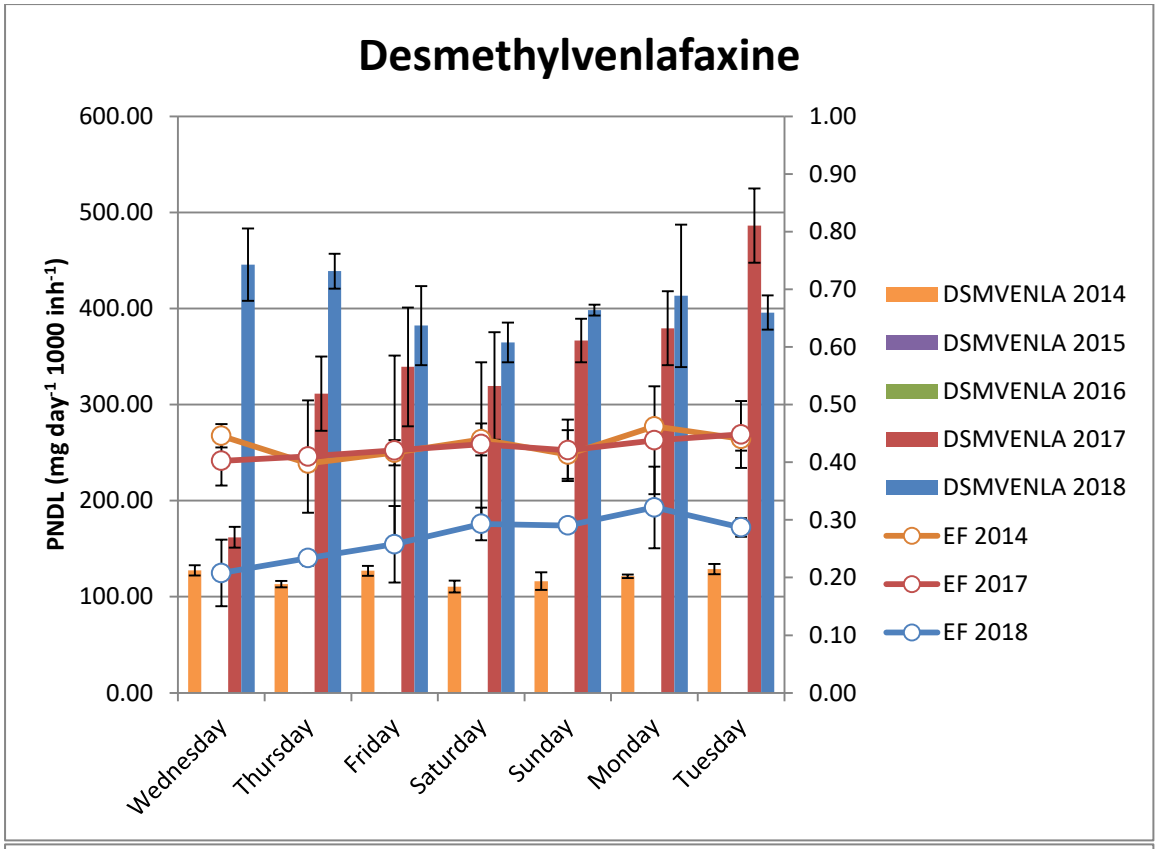

Ketamine
PNDL (mg day-1 1000 inh-1)
250.00
200.00
150.00
100.00
50.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
KETA 2014
KETA 2015
KETA 2016
KETA 2017
KETA 2018

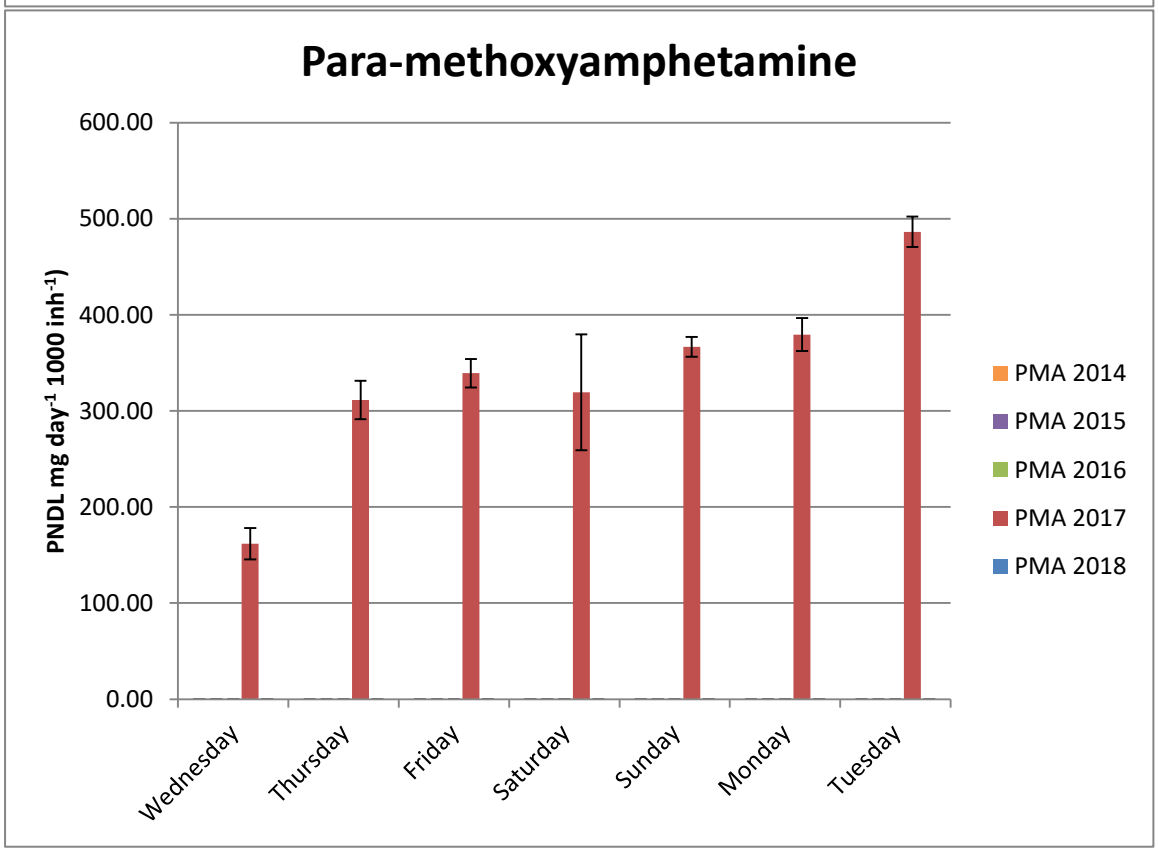

Vardenafil
PNDL (mg day-1 1000 inh-1)
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
VARDEN 2014
VARDEN 2015
VARDEN 2016
VARDEN 2017
VARDEN 2018

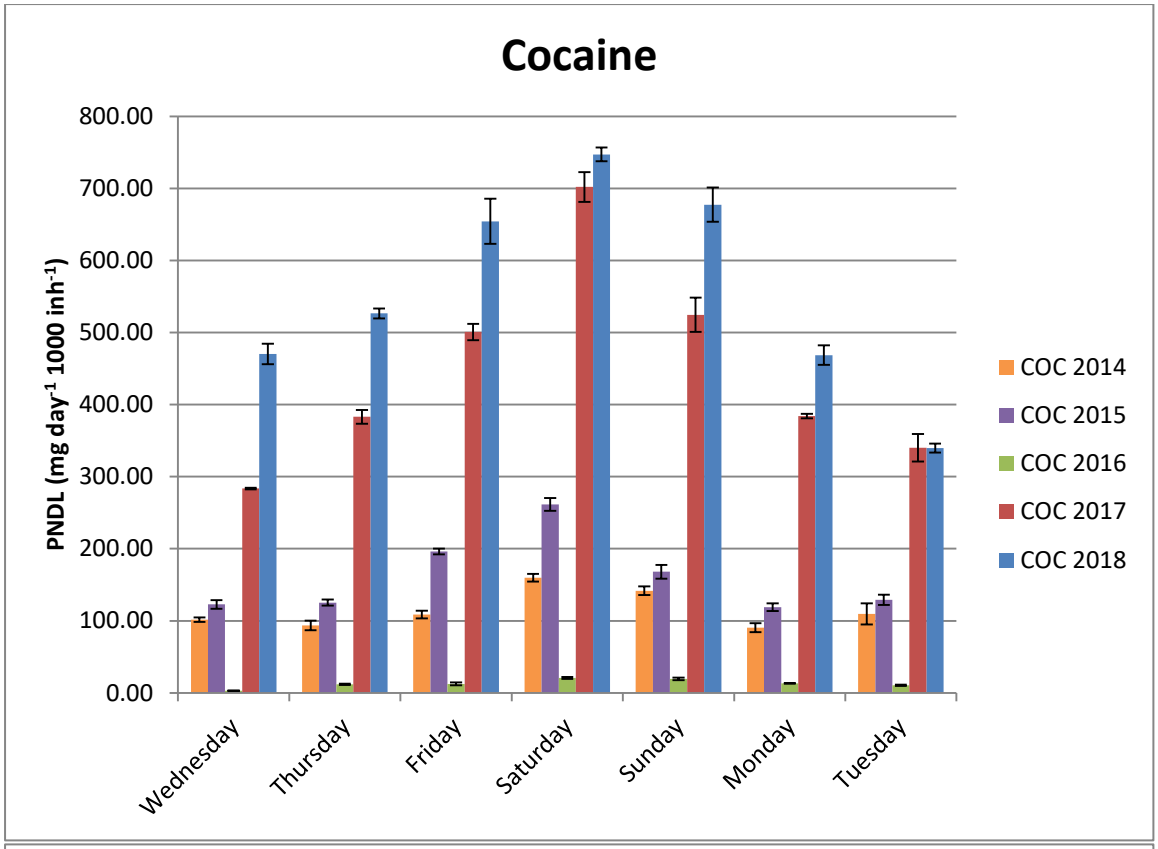

1R,2S-(-)-Ephedrine
PNDL (mg day-1 1000 inh-1)
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
EPH 2014
EPH 2015
EPH 2016
EPH 2017
EPH 2018

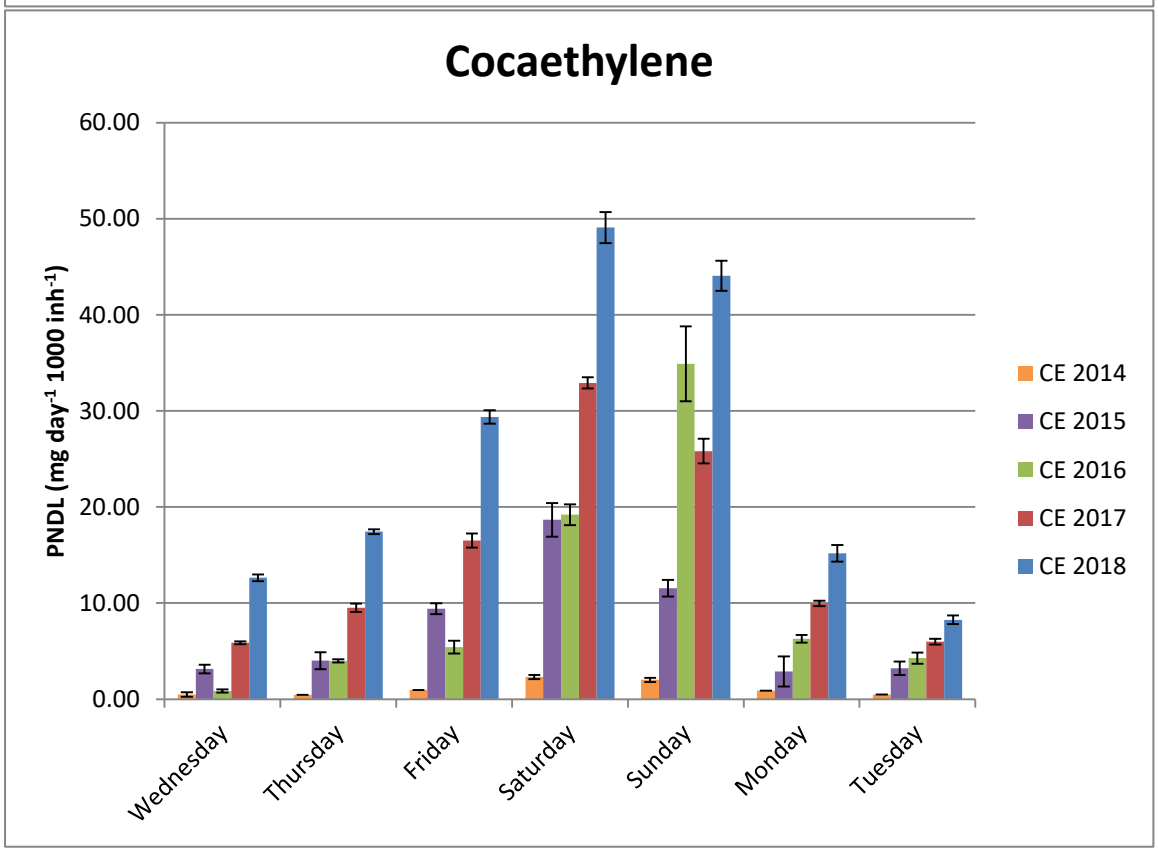

1S,2S-(+)-Pseudoephedrine
PNDL (mg day-1 1000 inh-1)
180.00
160.00
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
PSE 2014
PSE 2015
PSE 2016
PSE 2017
PSE 2018

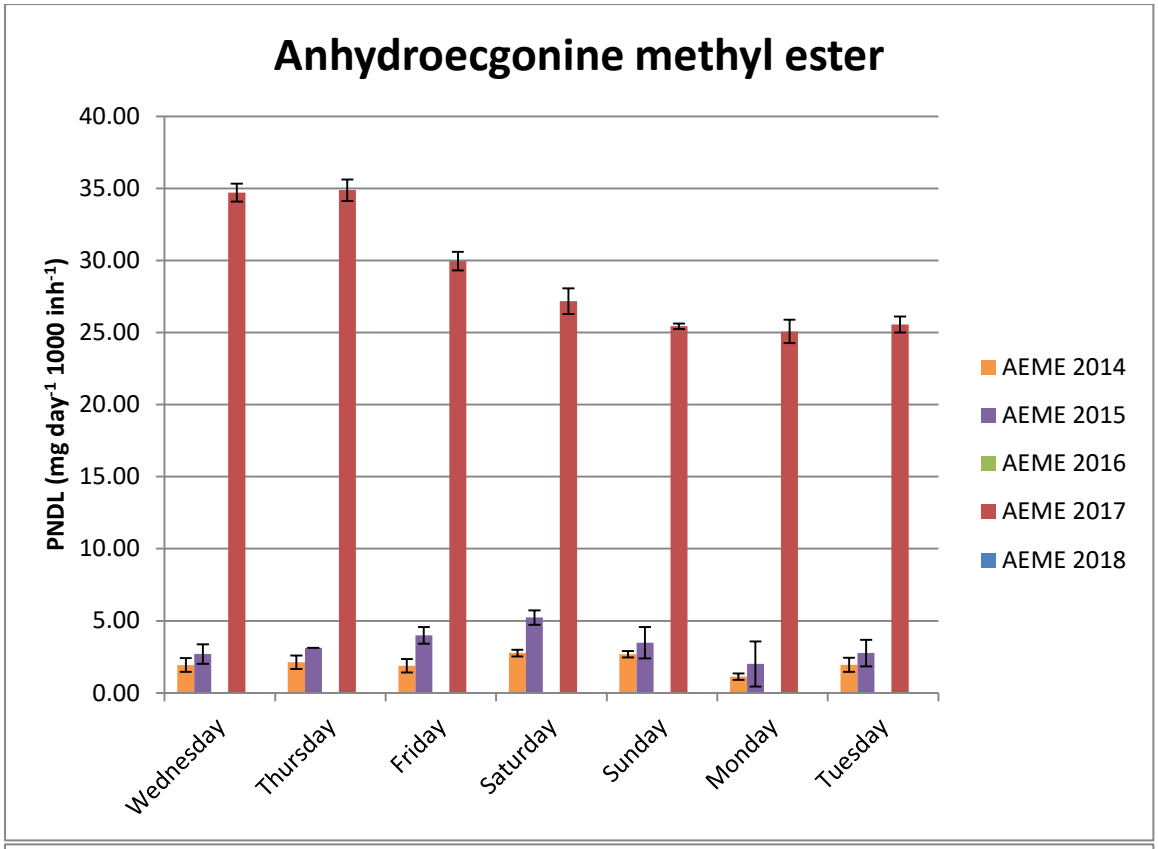

Norephedrine
PNDL (mg day-1 1000 inh-1)
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
NorEPH 2014
NorEPH 2015
NorEPH 2016
NorEPH 2017
NorEPH 2018

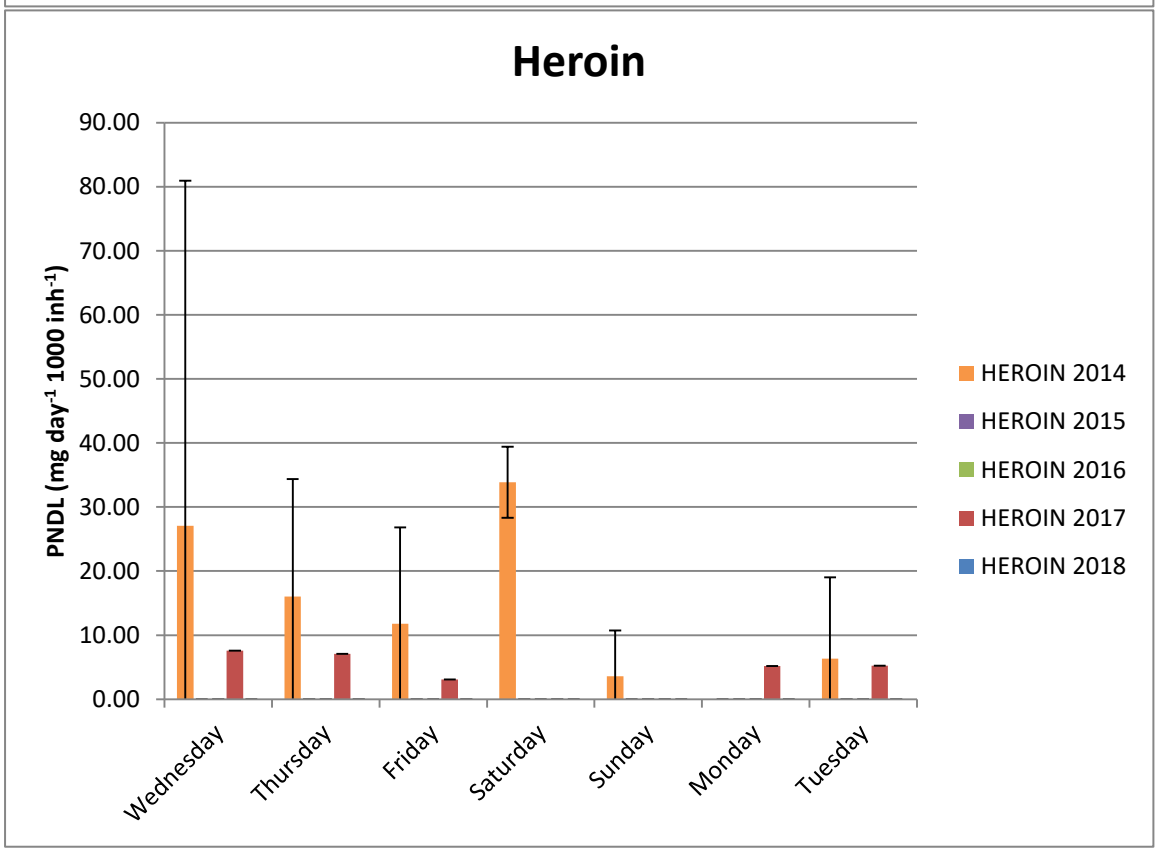

Desmethylvenlafaxine
PNDL (mg day-1 1000 inh-1)
600.00
500.00
400.00
300.00
200.00
100.00
0.00
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
DSMVENLA 2014
DSMVENLA 2015
DSMVENLA 2016
DSMVENLA 2017
DSMVENLA 2018
EF 2014
EF 2017
EF 2018

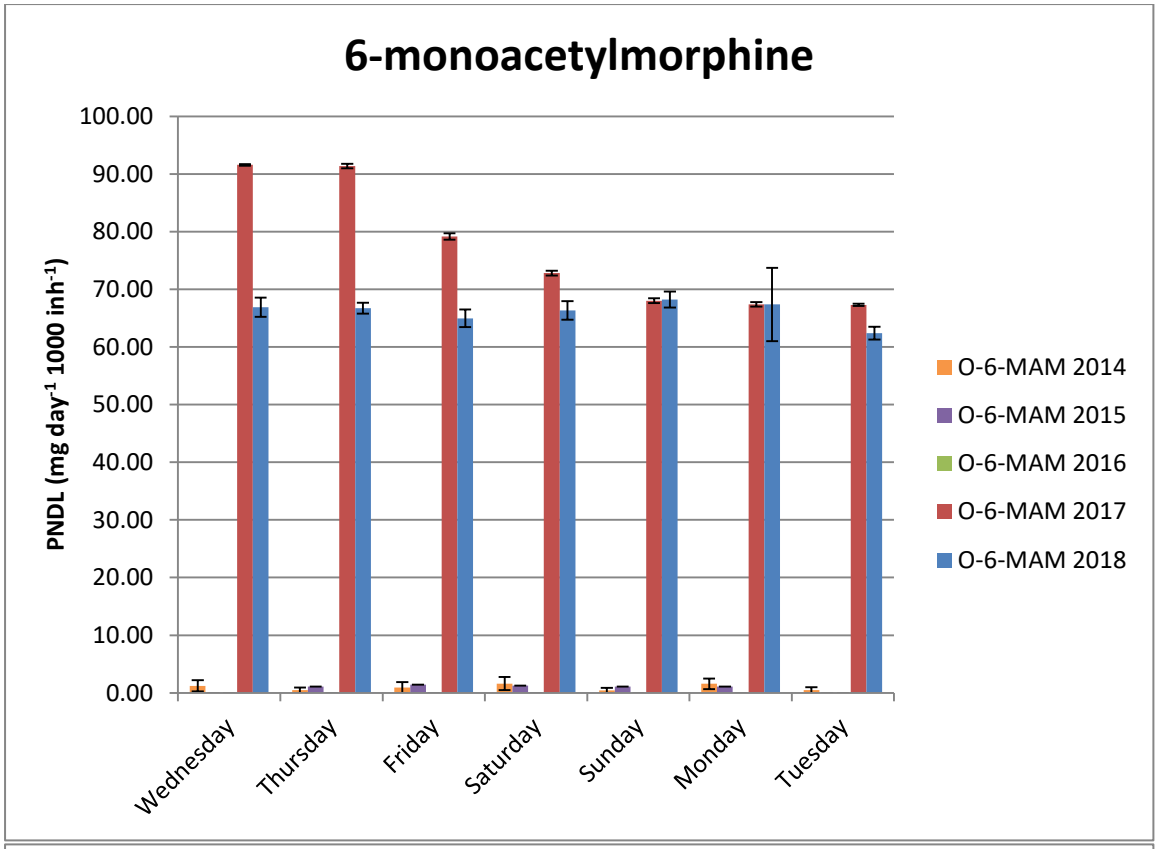

Para-methoxyamphetamine
PNDL mg day-1 1000 inh-1)
600.00
500.00
400.00
300.00
200.00
100.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
PMA 2014
PMA 2015
PMA 2016
PMA 2017
PMA 2018


Cocaine
PNDL (mg day-1 1000 inh-1)
800.00
700.00
600.00
500.00
400.00
300.00
200.00
100.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
COC 2014
COC 2015
COC 2016
COC 2017
COC 2018


Cocaethylene
PNDL (mg day-1 1000 inh-1)
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
CE 2014
CE 2015
CE 2016
CE 2017
CE 2018


Anhydroecgonine methyl ester
PNDL (mg day-1 1000 inh-1)
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
AEME 2014
AEME 2015
AEME 2016
AEME 2017
AEME 2018


Heroin
PNDL (mg day-1 1000 inh-1)
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
HEROIN 2014
HEROIN 2015
HEROIN 2016
HEROIN 2017
HEROIN 2018


6-monoacetylmorphine
PNDL (mg day-1 1000 inh-1)
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
O-6-MAM 2014
O-6-MAM 2015
O-6-MAM 2016
O-6-MAM 2017
O-6-MAM 2018

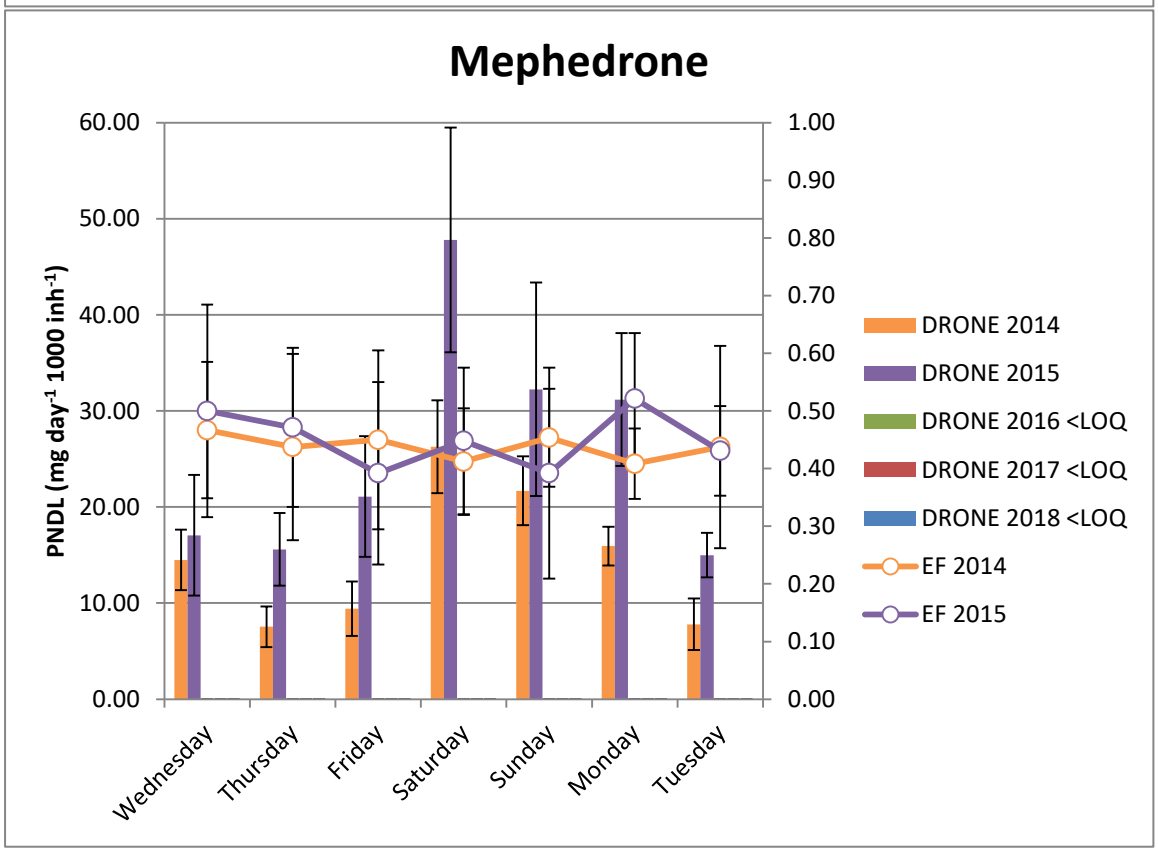
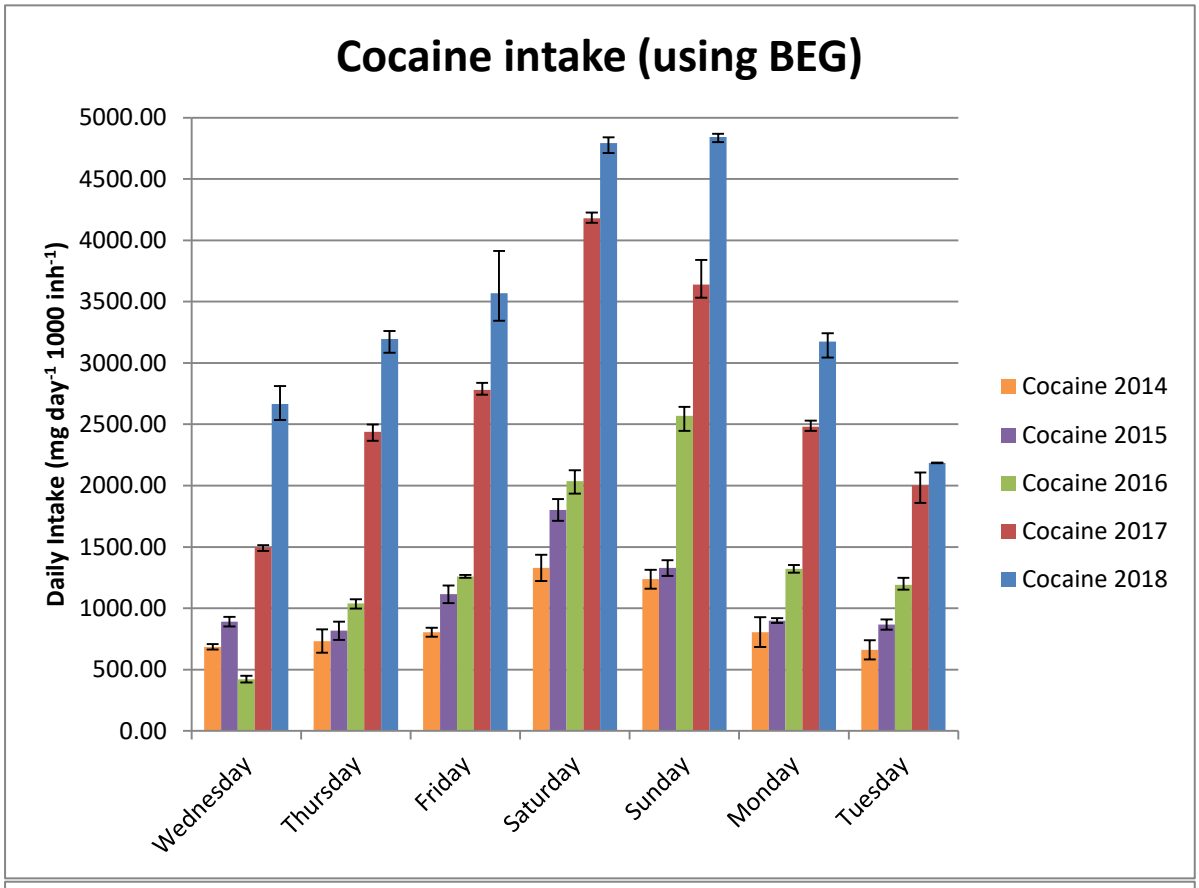
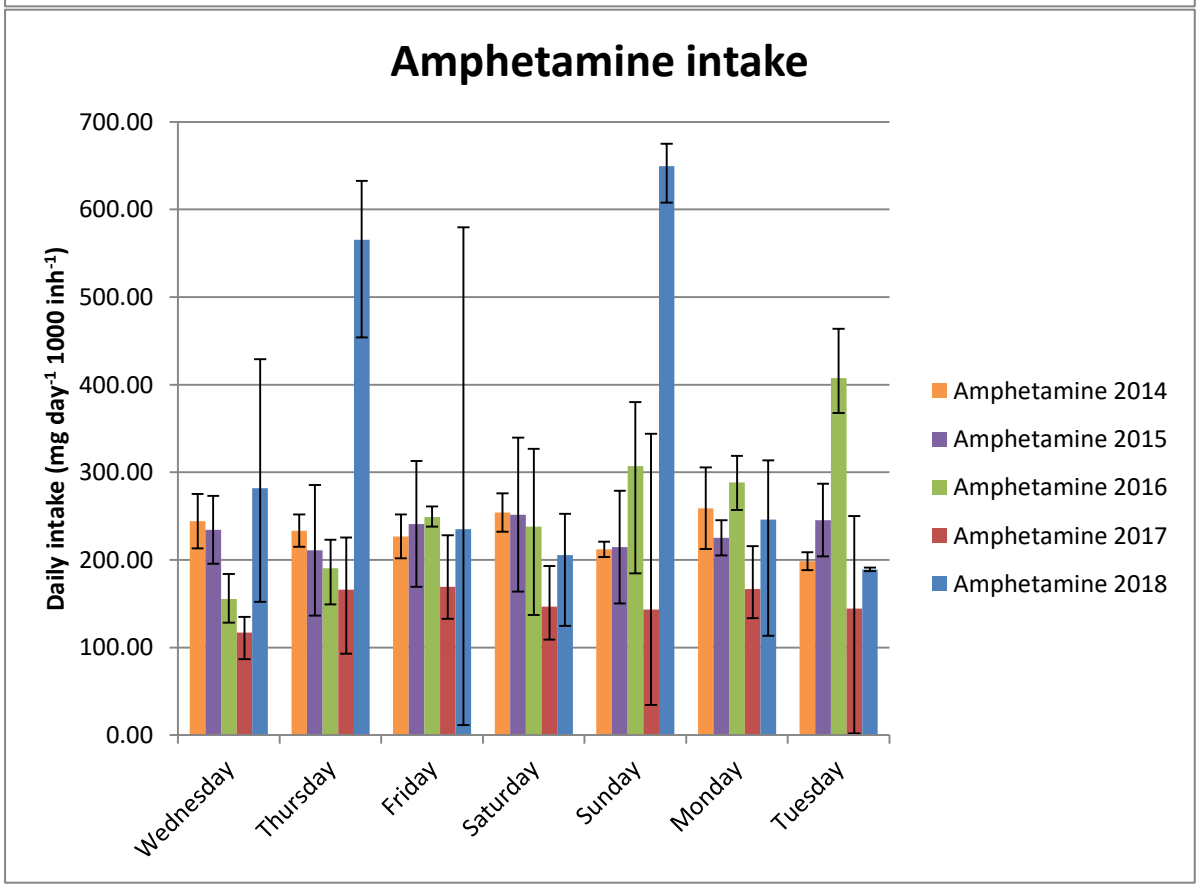
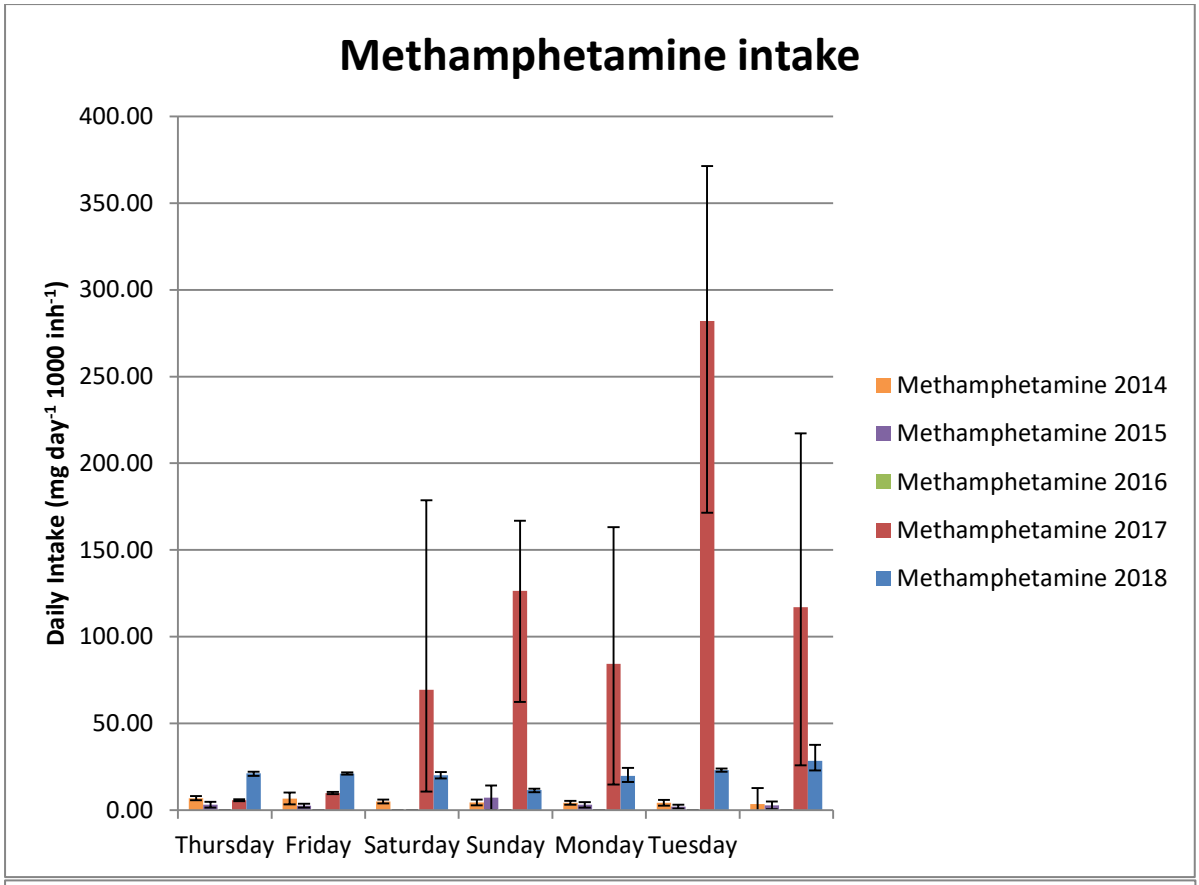
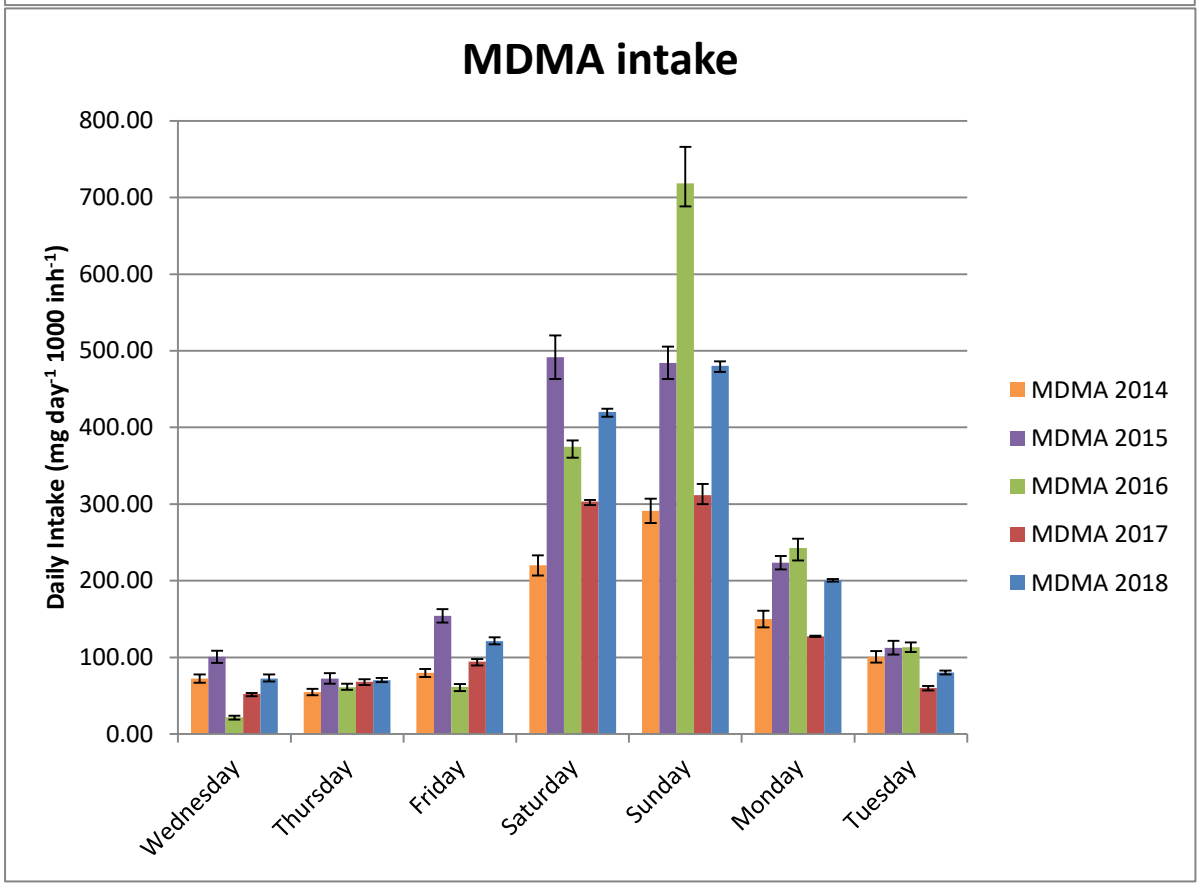

Mephedrone
PNDL (mg day-1 1000 inh-1)
60.00
50.00
40.00
30.00
20.00
10.00
0.00
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
DRONE 2014
DRONE 2015
DRONE 2016 <LOQ
DRONE 2017 <LOQ
DRONE 2018 <LOQ
EF 2014
EF 2015

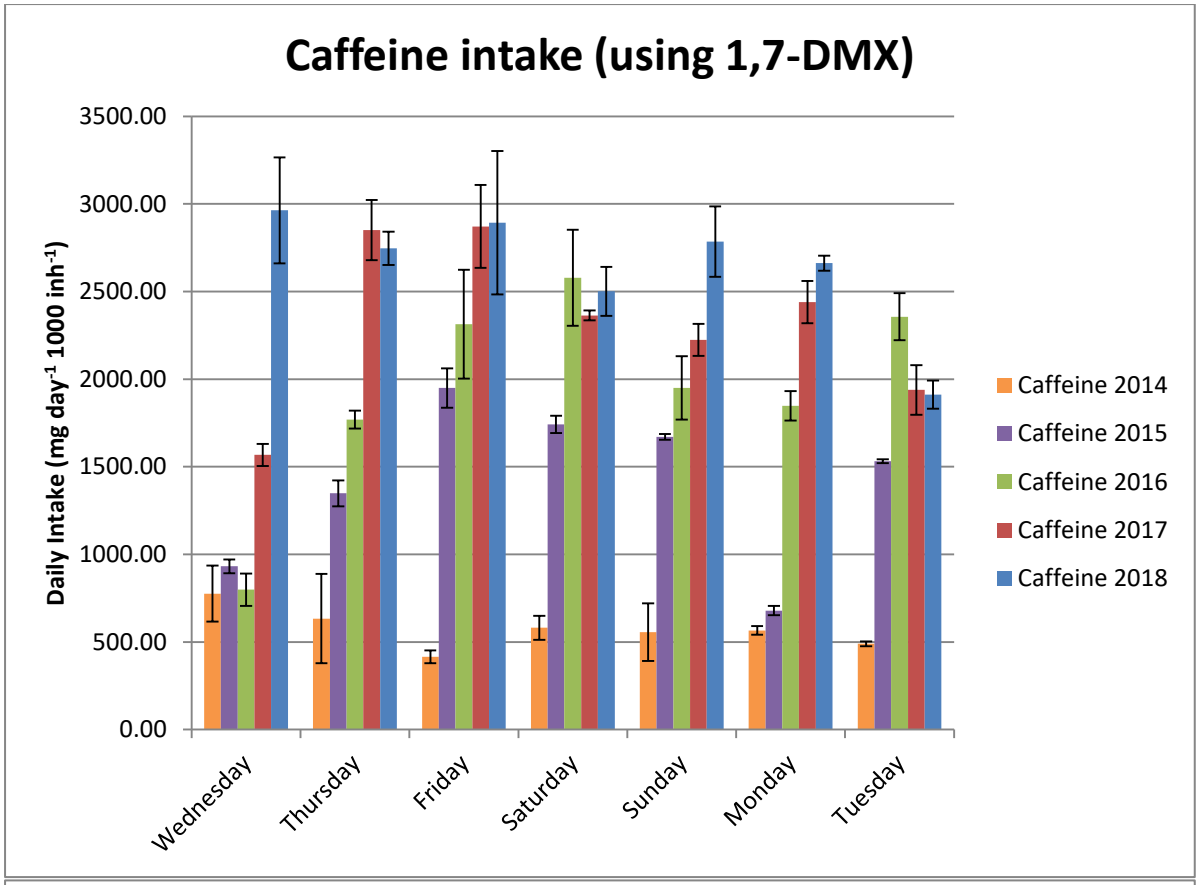
Figure S2. Daily intake

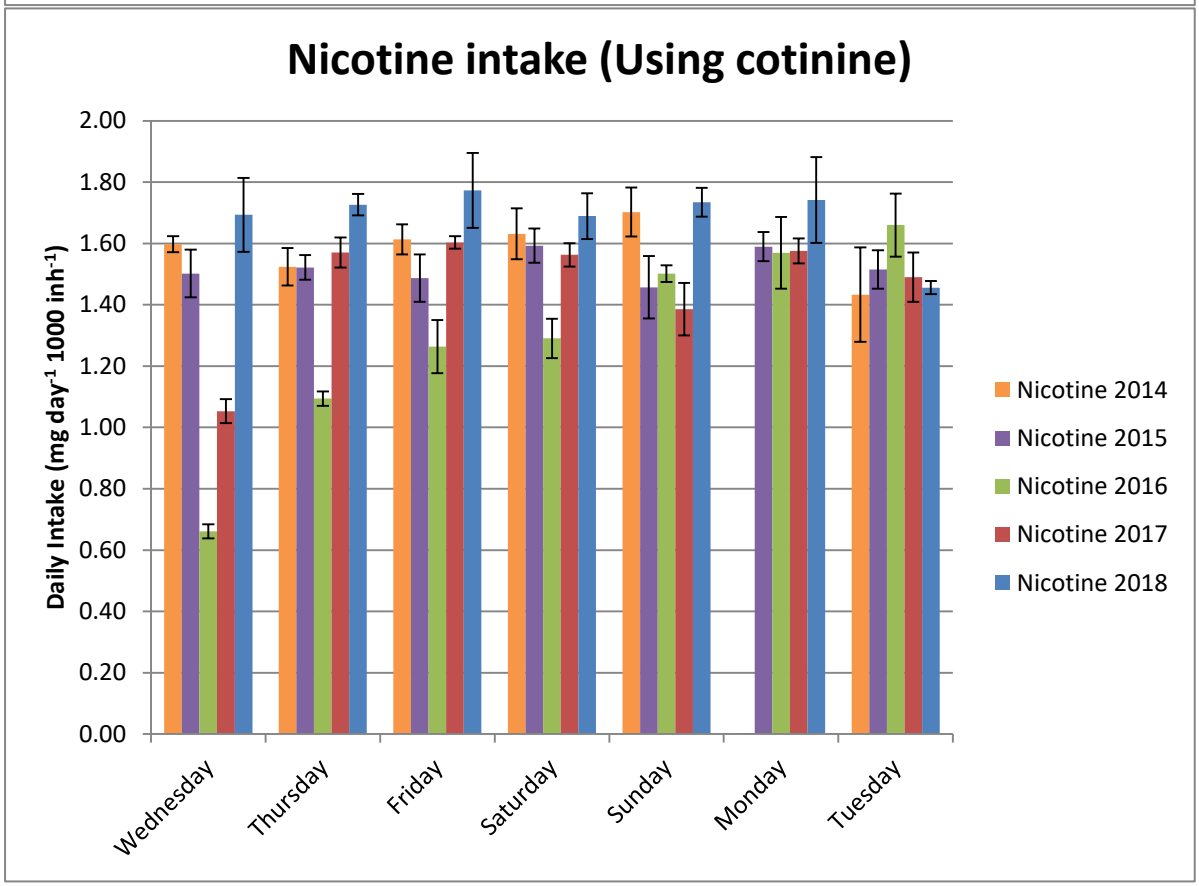


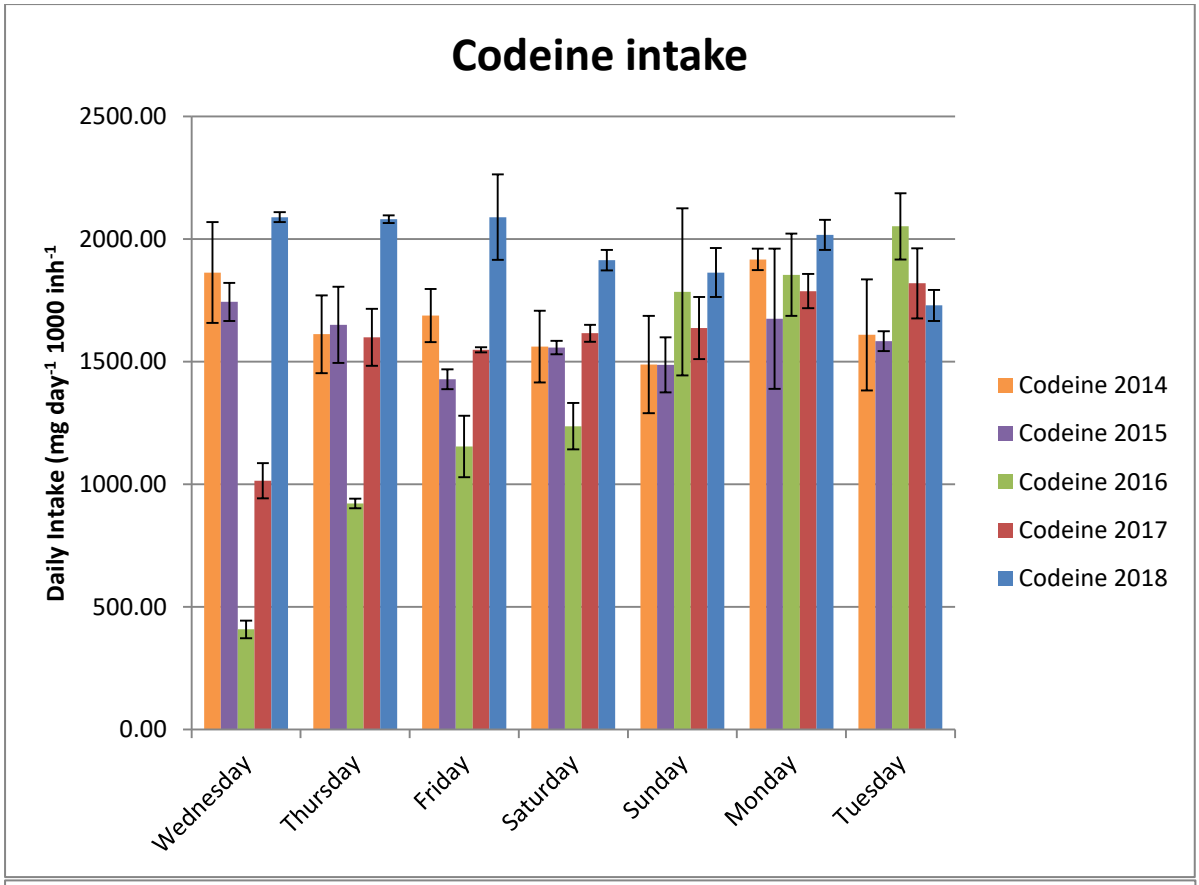


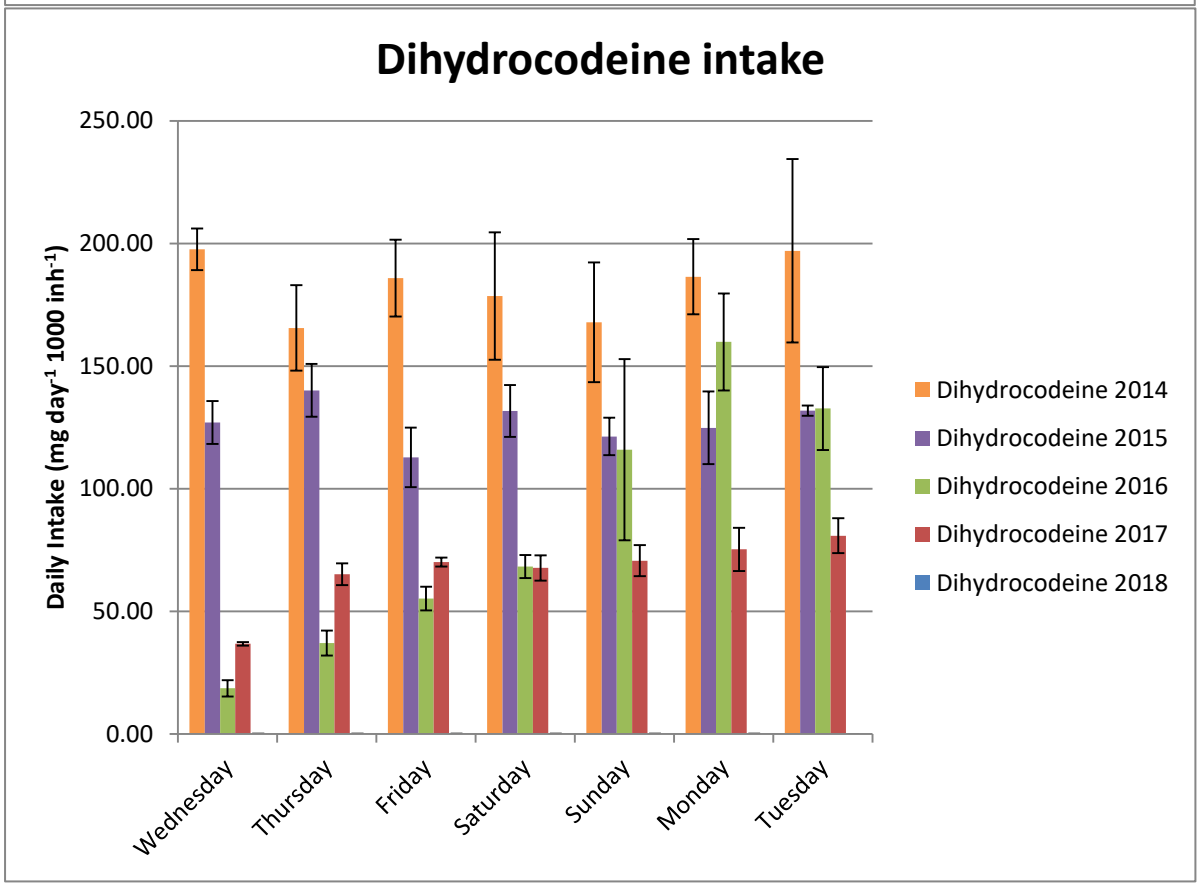

Methamphetamine intake
Daily Intake (mg day-1 1000 inh-1)
400.00
350.00
300.00
250.00
200.00
150.00
100.00
50.00
0.00
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
Methamphetamine 2014
Methamphetamine 2015
Methamphetamine 2016
Methamphetamine 2017
Methamphetamine 2018

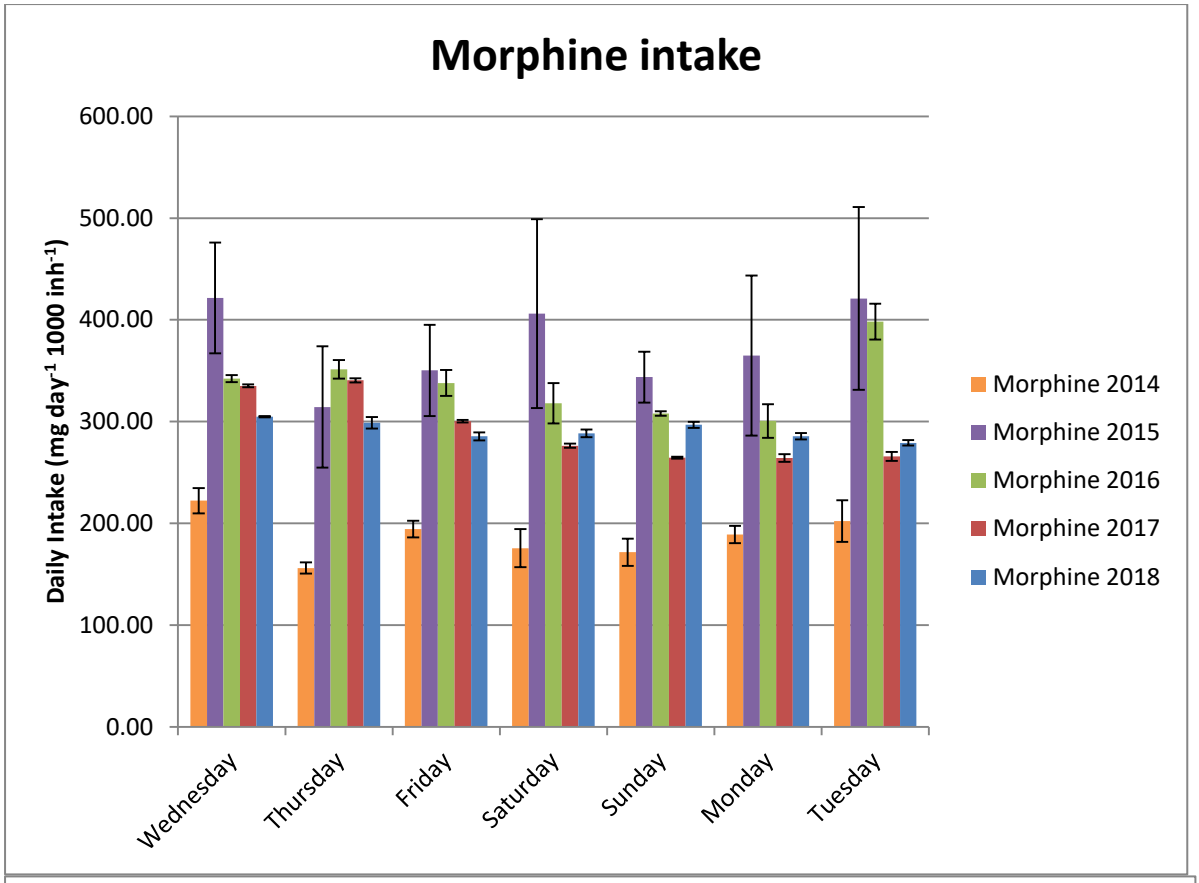

MDMA intake
Daily Intake (mg day-1 1000 inh-1)
800.00
700.00
600.00
500.00
400.00
300.00
200.00
100.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
MDMA 2014
MDMA 2015
MDMA 2016
MDMA 2017
MDMA 2018

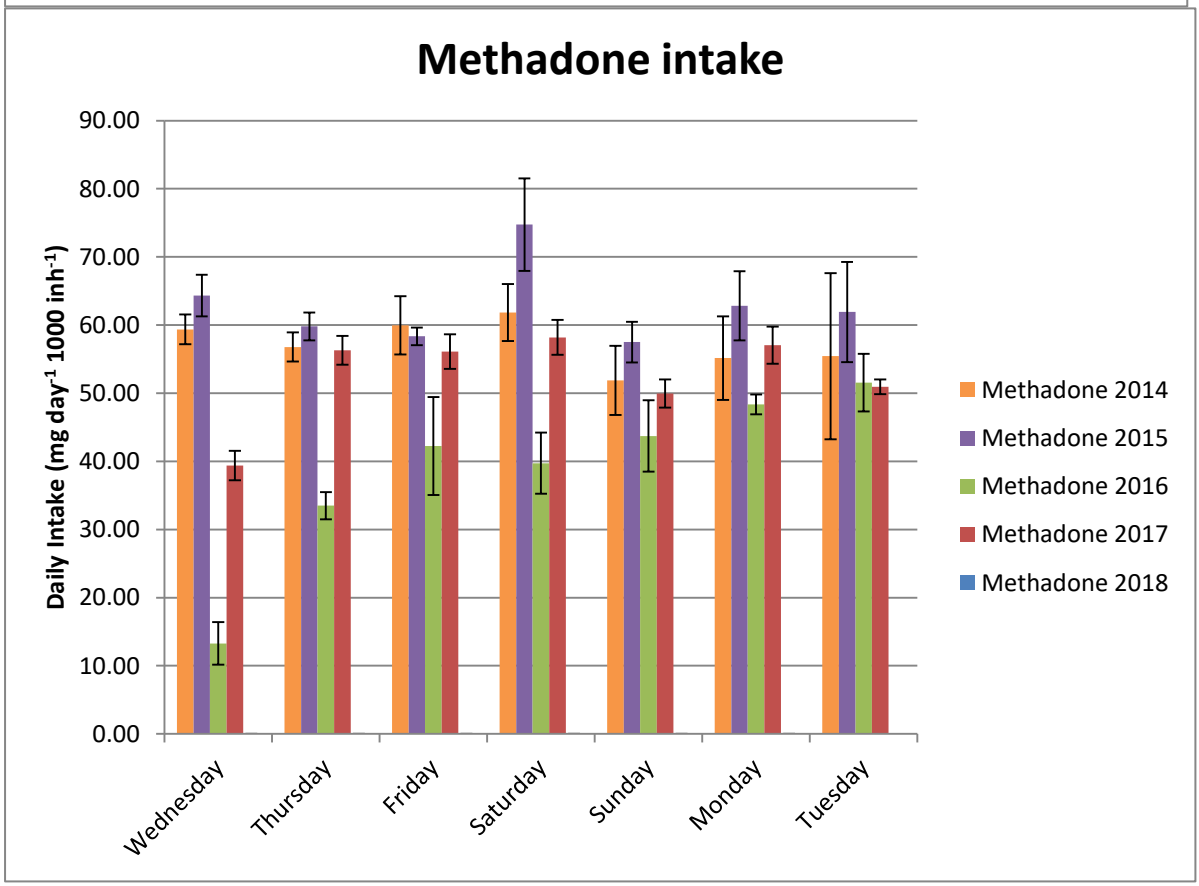

Caffeine intake (using 1,7-DMX)
Daily Intake (mg day-1 1000 inh-1)
3500.00
3000.00
2500.00
2000.00
1500.00
1000.00
500.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
Caffeine 2014
Caffeine 2015
Caffeine 2016
Caffeine 2017
Caffeine 2018

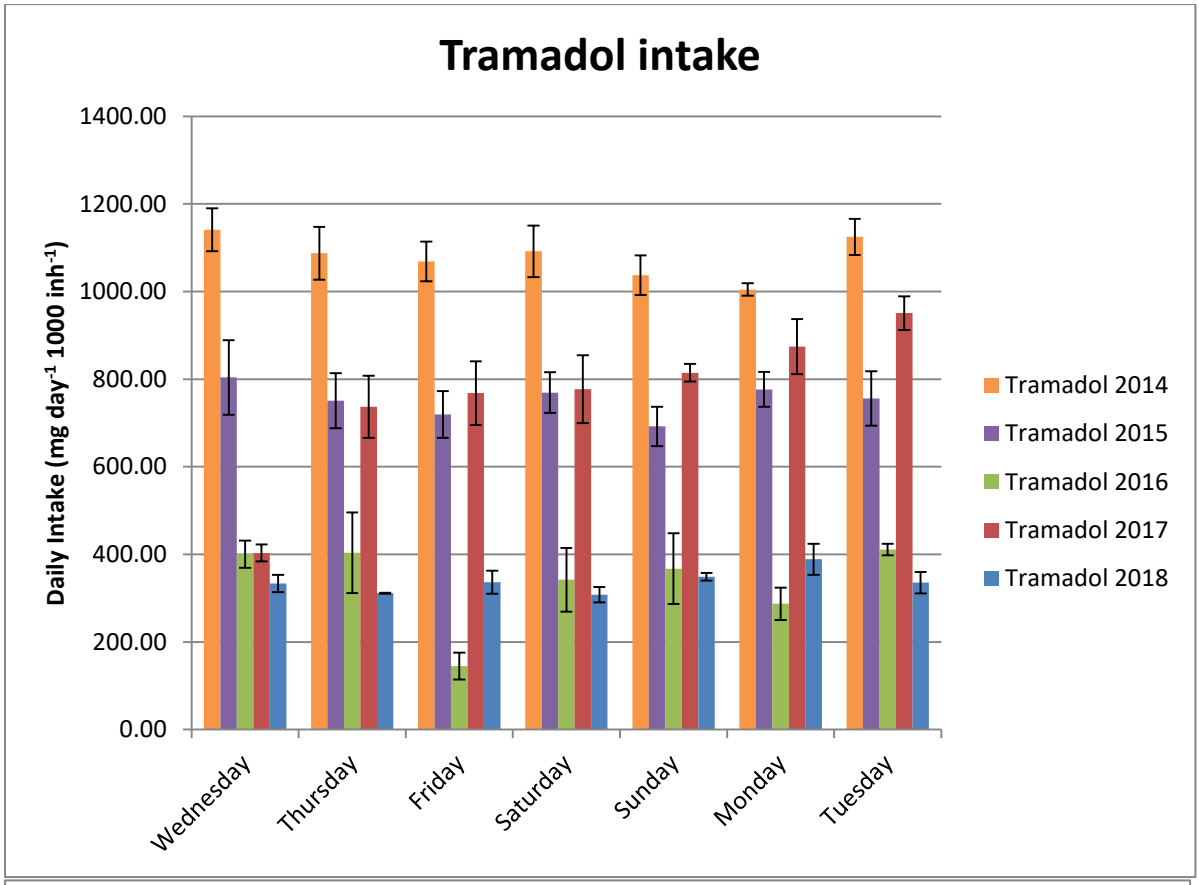

Nicotine intake (Using cotinine)
Daily Intake (mg day-1 1000 inh-1)
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
Nicotine 2014
Nicotine 2015
Nicotine 2016
Nicotine 2017
Nicotine 2018

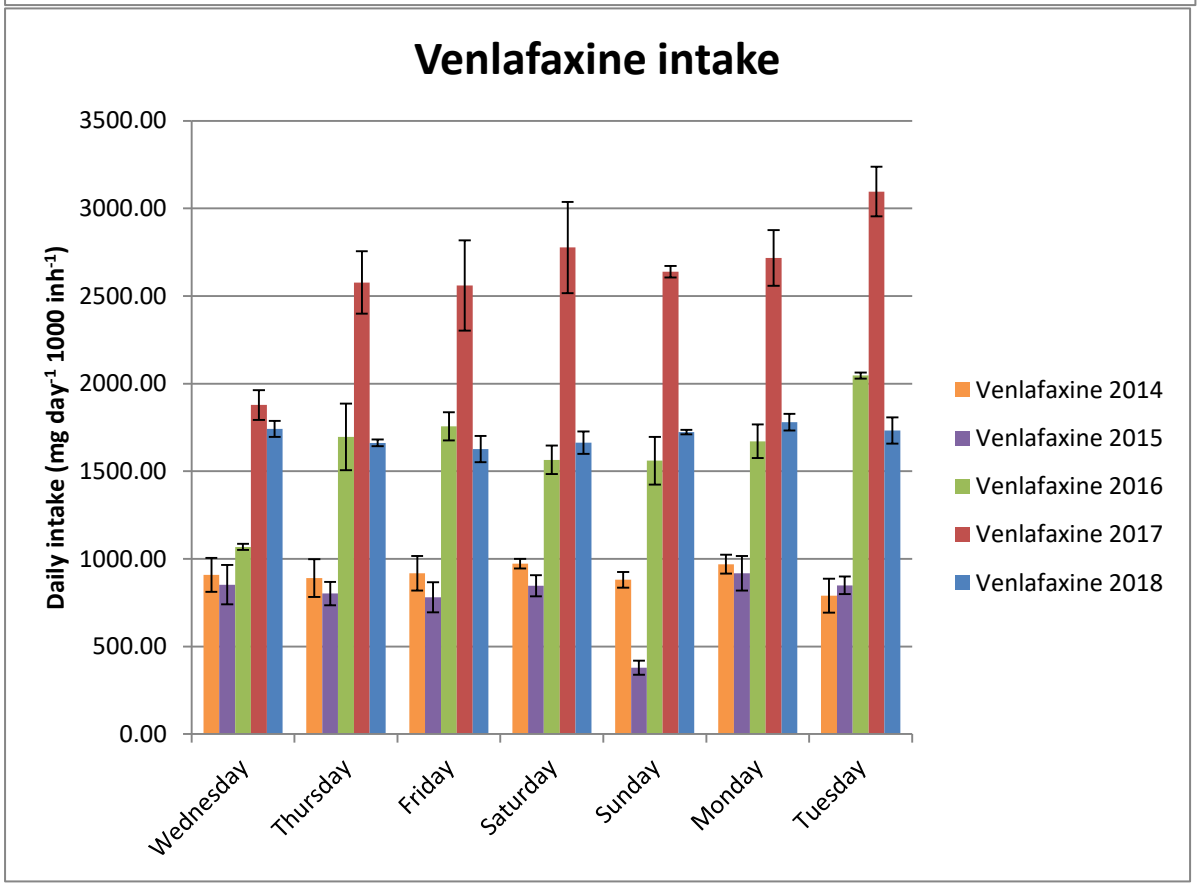

Codeine intake
Daily Intake (mg day-1 1000 inh-1
2500.00
2000.00
1500.00
1000.00
500.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
Codeine 2014
Codeine 2015
Codeine 2016
Codeine 2017
Codeine 2018

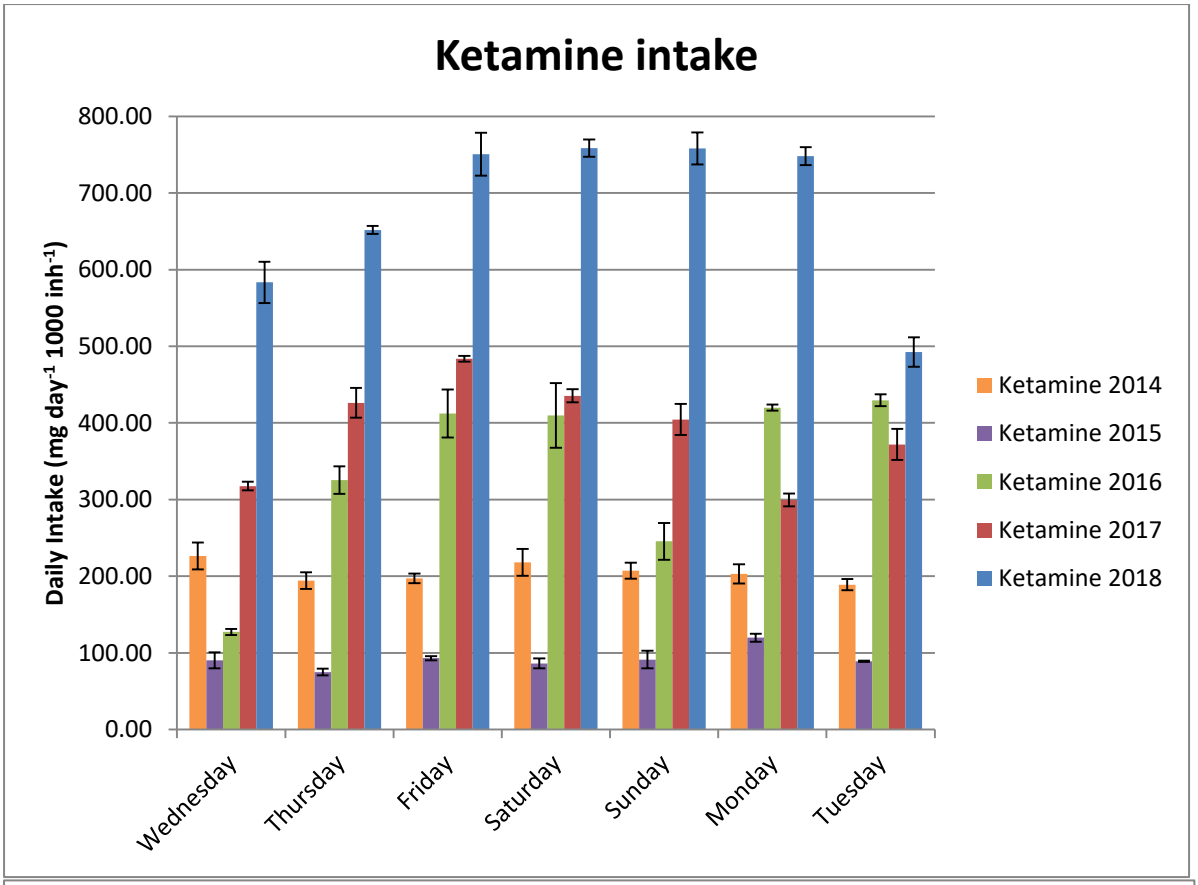

Dihydrocodeine intake
Daily Intake (mg day-1 1000 inh-1)
250.00
200.00
150.00
100.00
50.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
Dihydrocodeine 2014
Dihydrocodeine 2015
Dihydrocodeine 2016
Dihydrocodeine 2017
Dihydrocodeine 2018

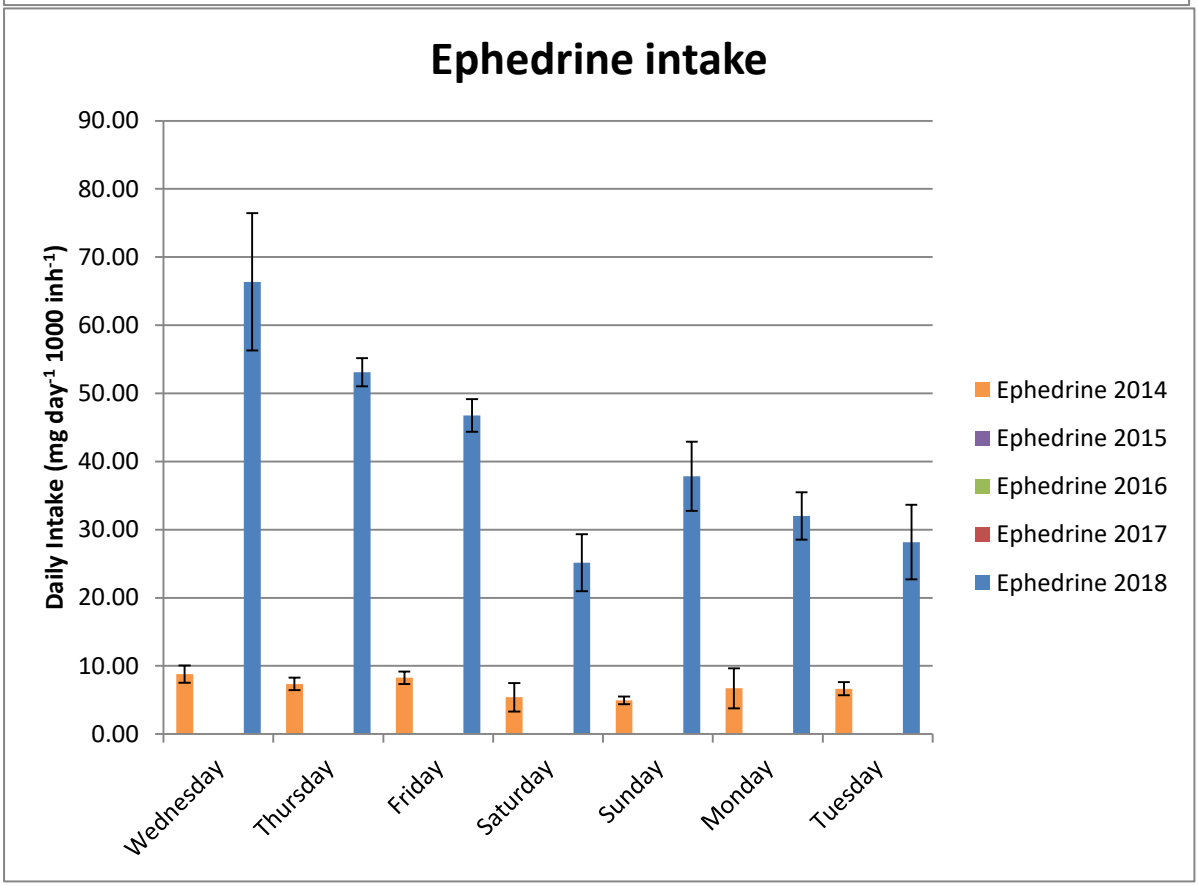

Morphine intake
Daily Intake (mg day-1 1000 inh-1)
600.00
500.00
400.00
300.00
200.00
100.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
Morphine 2014
Morphine 2015
Morphine 2016
Morphine 2017
Morphine 2018

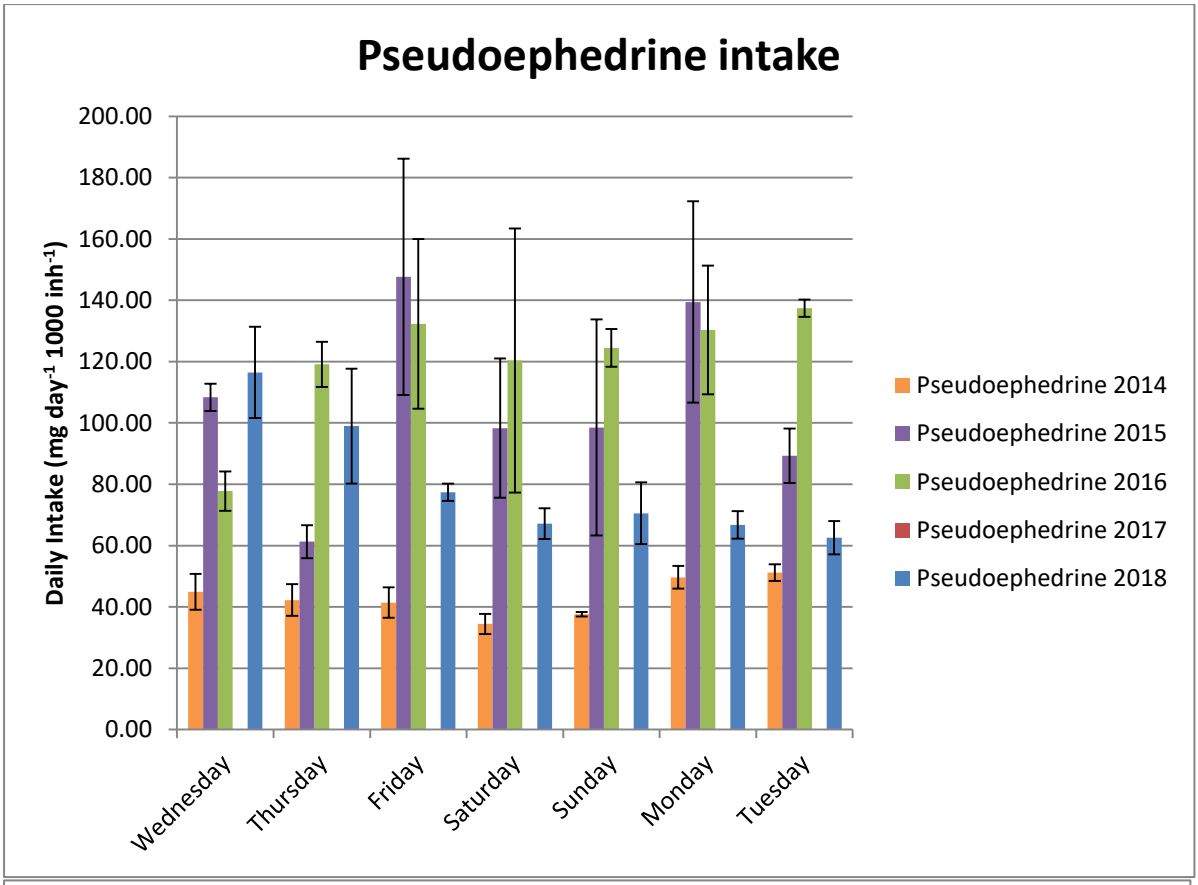

Methadone intake
Daily Intake (mg day-1 1000 inh-1)
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
Methadone 2014
Methadone 2015
Methadone 2016
Methadone 2017
Methadone 2018

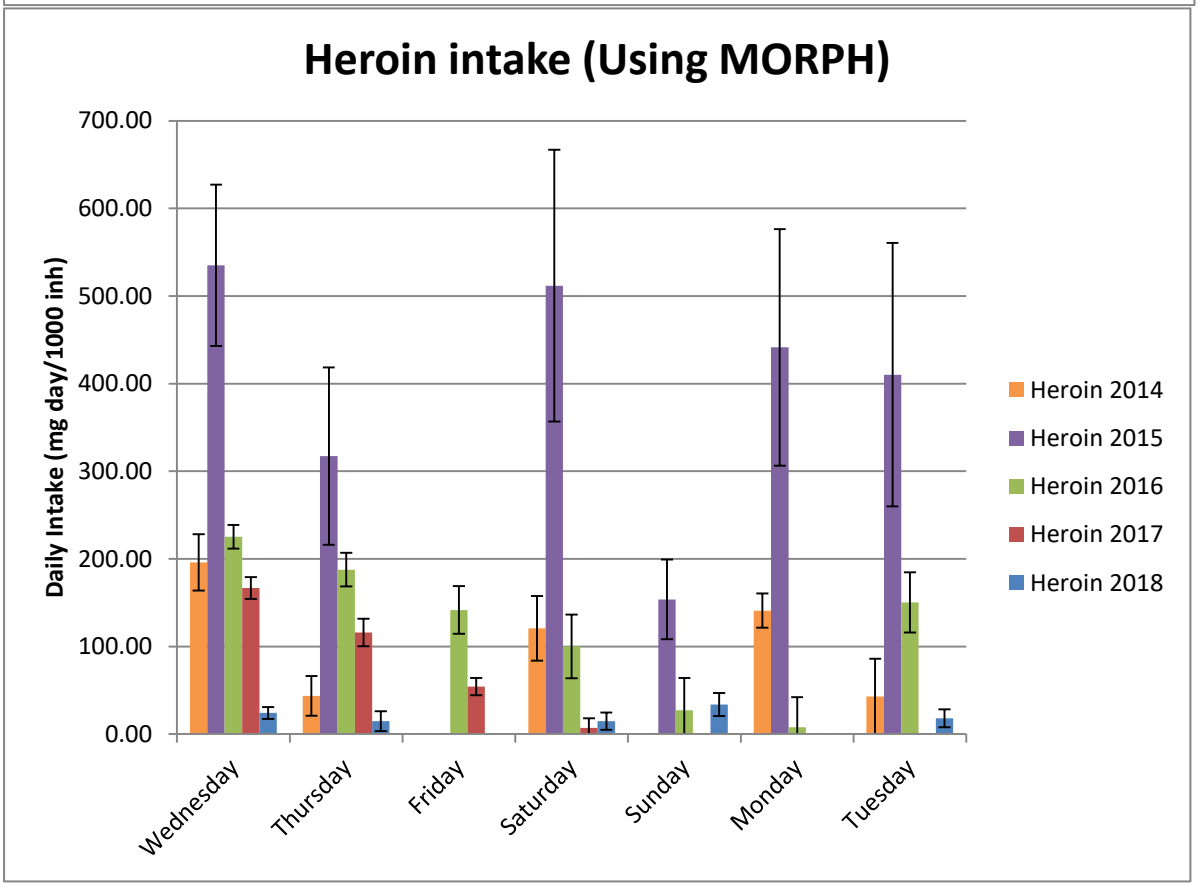

Tramadol intake
Daily Intake (mg day-1 1000 inh-1)
1400.00
1200.00
1000.00
800.00
600.00
400.00
200.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
Tramadol 2014
Tramadol 2015
Tramadol 2016
Tramadol 2017
Tramadol 2018

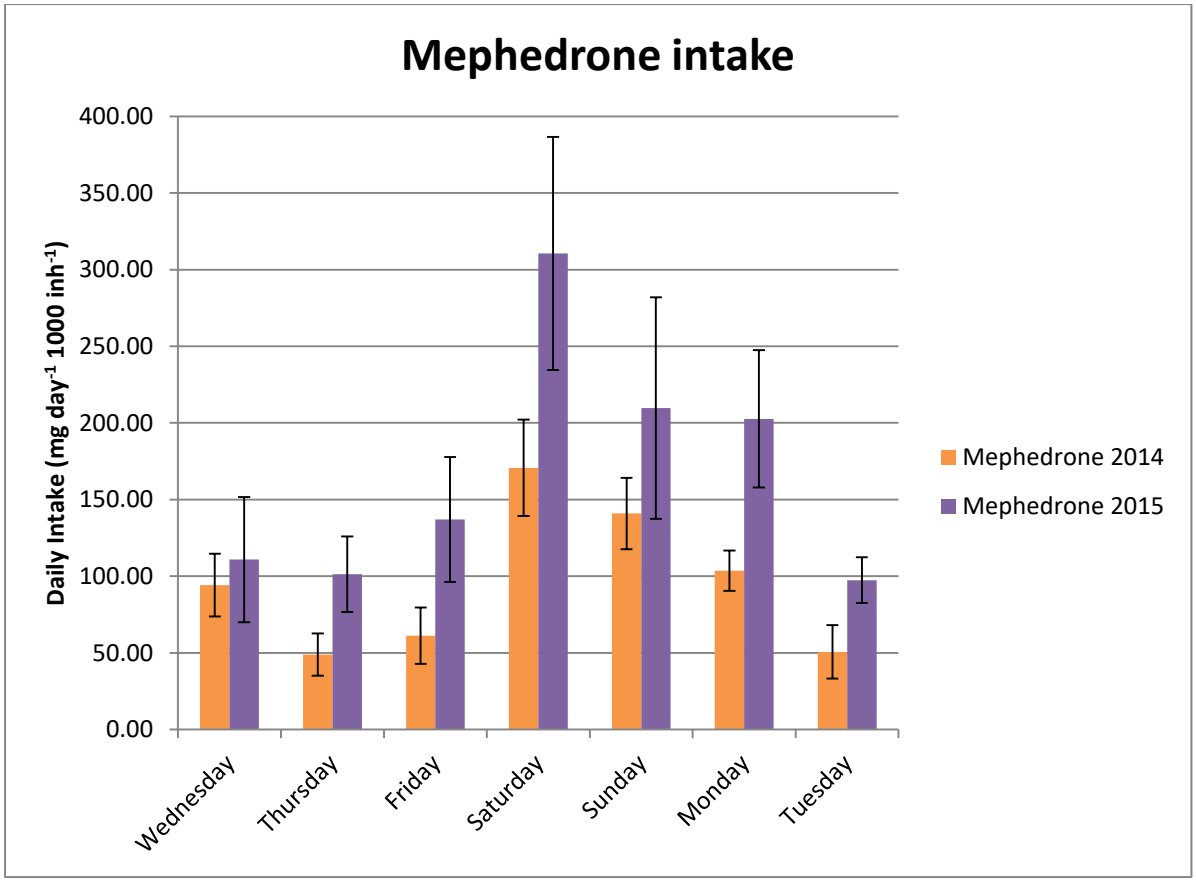

Venlafaxine intake
Daily intake (mg day-1 1000 inh-1)
3500.00
3000.00
2500.00
2000.00
1500.00
1000.00
500.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
Venlafaxine 2014
Venlafaxine 2015
Venlafaxine 2016
Venlafaxine 2017
Venlafaxine 2018


Ketamine intake
Daily Intake (mg day-1 1000 inh-1)
800.00
700.00
600.00
500.00
400.00
300.00
200.00
100.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
Ketamine 2014
Ketamine 2015
Ketamine 2016
Ketamine 2017
Ketamine 2018


Ephedrine intake
Daily Intake (mg day-1 1000 inh-1)
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
Ephedrine 2014
Ephedrine 2015
Ephedrine 2016
Ephedrine 2017
Ephedrine 2018


Pseudoephedrine intake
Daily Intake (mg day-1 1000 inh-1)
200.00
180.00
160.00
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
Pseudoephedrine 2014
Pseudoephedrine 2015
Pseudoephedrine 2016
Pseudoephedrine 2017
Pseudoephedrine 2018


Heroin intake (Using MORPH)
Daily Intake (mg day/1000 inh)
700.00
600.00
500.00
400.00
300.00
200.00
100.00
0.00
Wednesday
Thursday
Friday
Saturday
Sunday
Monday
Tuesday
Heroin 2014
Heroin 2015
Heroin 2016
Heroin 2017
Heroin 2018



Lifestyle chemicals

Caffeine PNDLs did not show much change over the study period only increasing significantly from 2014-2015 but caffeine was not consistently used across the week (Fig S1-2), with weekly significant variation every year. Caffeine's major urinary metabolite, 1,7-dimethylxanthine (1,7-DMX), followed the same overall pattern of stable usage year on year, except from 2014-2015 where there was no significant change in PNDLs of 1,7-DMX. Like with caffeine there was no clear weekly pattern of excretion (Fig S3).

Nicotine usage was similarly static across the study period with significant increase only between 2015 and 2016 and significant weekly variation observed for every year except 2014 (Fig S3). Trends in PNDLs of cotinine, the main urinary nicotine metabolite, were broadly similar as could be expected of a metabolite. The only significant increase in loads of cotinine was between 2017 and 2018, and there was significant weekly variation for every year except 2015. Significant public health initiatives are centred on reducing rates of smoking and data shows that the prevalence of smoking is decreasing [ONS report]. Between 2014 and 2017 the percentage of adults who smoked in the United Kingdom decreased from 18.1% to 15.1%, with our study city having a below average result of 11.1% in 2017. Similarly, the percentage of people using electronic cigarettes (e-cigarettes) has increased from 3.7% in

2014 to 5.5% in 2017. Whilst smokers and e-cigarettes users are not mutually exclusive groups the increased use of e-cigarettes could explain why nicotine and cotinine loads are not decreasing despite the number of adults who smoke decreasing. This could potentially point to increasing rates of smoking among a decreasing population, but further data on consumer spending would be required to determine this as current data only asks if a participant smokes and not how much. Alternatively, as the amount of nicotine per dose/puff of an e-cigarette can be programmed, then users could potentially be consuming more nicotine and so contributing more to wastewater loads than a tobacco smoker. Again more data would be required to determine an exact cause of this discrepancy but in either case this highlights the power of WBE for understanding the reality of what is being consumed rather than the more abstract model of extrapolating individual trends to understand the whole population, which is important for determining if government health strategies are effective.

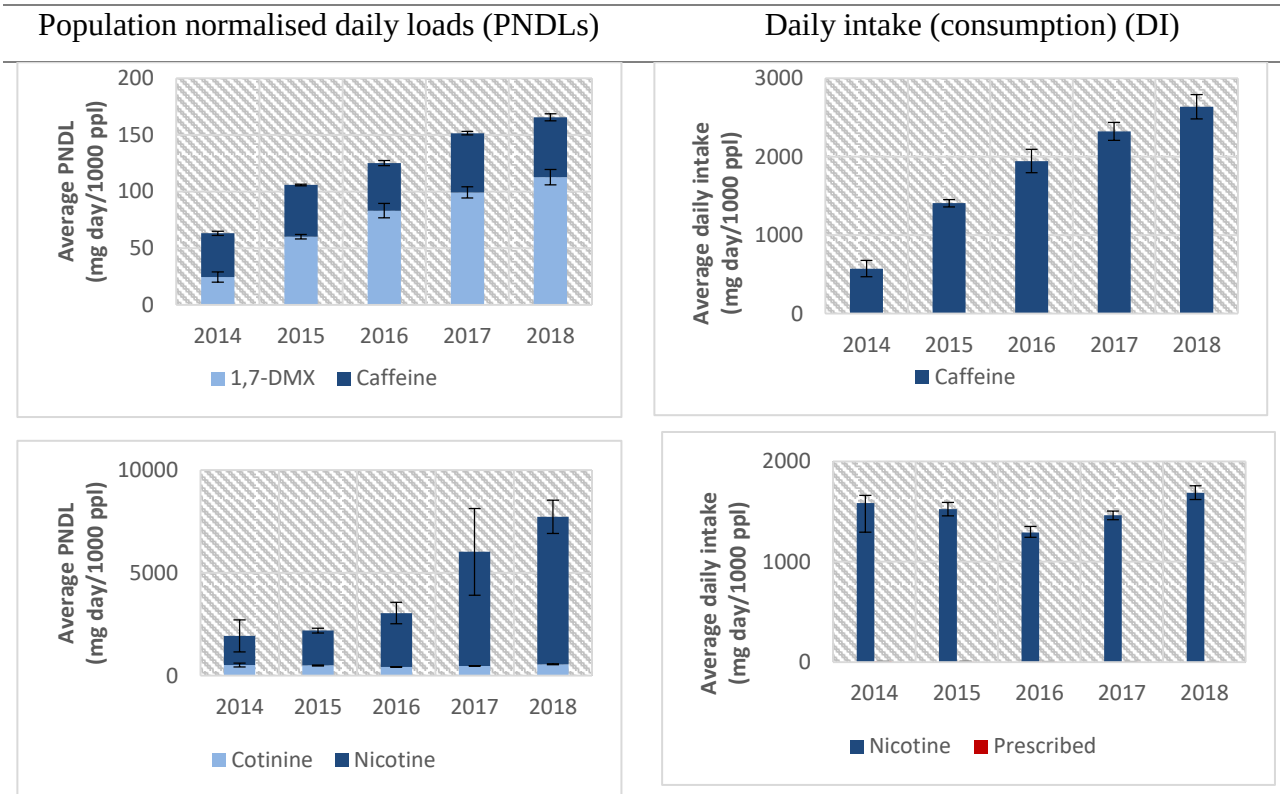
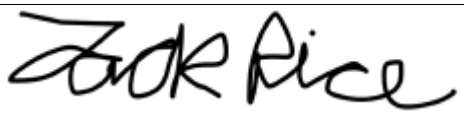


Figure S3. Average PNDL and DI of (i) caffeine and its metabolite 1,7-DMX, and (ii) nicotine and its metabolite cotinine.

This declaration concerns the article entitled:	
Stereochemistry of ephedrine and its environmental significance: Exposure and effects directed approach	
Publication status (tick one) **Draft manuscript** [ ] **Submitted** [ ] **In review** [ ] **Accepted** [ ] **Published** [X]	
Publication details (reference)	DOI: 10.1016/j.jhazmat.2018.01.020 Rice, J., Proctor, K., Lopardo, L., Evans, S., Kasprzyk-Hordern, B., 2018. Stereochemistry of ephedrine and its environmental significance: Exposure and effects directed approach, *Journal of Hazardous Materials*, 348, pp 39-46
Copyright status (tick the appropriate statement) I hold the copyright for this material [ ] Copyright is retained by the publisher, but I have been given permission to replicate the material here [X]	
Candidate's contribution to the paper (provide details, and also indicate as a percentage)	The candidate contributed to / considerably contributed to / predominantly executed the… Formulation of ideas: . Candidate contributed to the formulation of ideas for the microcosm and D. magna toxicity experiments in collaboration with Barbara Kasprzyk-Hordern (BKH) and Sian Evans (SEE) .Candidate significantly contributed to the formulation of ideas for the HLM experiments in collaboration with Luigi Lopardo and BKH Design of methodology: . SEE predominantly executed the validation of the analytical method and the design of the microcosm method used . Candidate considerably contributed to the design of the specific microcosm experiment performed in the paper in collaboration with SEE . Candidate considerably contributed to the design of the *Daphnia magna t*oxicity experiments in collaboration with SEE and BKH . SEE predominantly executed the design of the Pseudokirchneriella subcapitata experiments . Kathryn Proctor predominantly executed the design of the *Tetrahymena thermophila* . LL considerably contributed to the development and validation of the human liver microsome (HLM) methodology

	. Candidate considerably contributed to the design of the specific HLM experiments performed in the paper in collaboration with LL Experimental work: . Candidate predominantly executed the microcosm experiments performed, in collaboration with SEE . Candidate predominantly executed to the analysis and interpretation of microcosm experiments, in collaboration with SEE . Candidate considerably contributed to the D. magna toxicity tests performed, in collaboration with SEE . Candidate considerably contributed to the HLM experiments performed, in collaboration with LL . Candidate predominantly executed the analysis and interpretation of HLM experiments, in collaboration with LL . SEE predominantly executed the P. subcapitata experiments performed . KP predominantly executed the T. thermophile experiments performed Presentation of data in journal format: Candidate predominantly executed the writing and formatting of all data for publication KP contributed to writing the section concerning T. thermophile and formatting related data LL contributed to writing the section concerning HLM		
Statement from Candidate	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature.		
Signed		**Date**	02/06/2020

Chapter two introduction

In chapter one the was focus was on introducing the use of local prescription data for providing context to wastewater data and showcasing the importance of chirality in determining the source of drugs of abuse and confirming the human consumption of analytes. In chapter two the narrative changes from an examination of human health to investigating environmental health by investigating the environmental stability and toxicity of the four isomers of ephedrine. As such, chapter two directly explores the first aim of the thesis as laid out in the introduction, including an assessment of chirality for determining the source of an analyte.

The objective of chapter two was to examine the stability of each isomer of ephedrine under environmental conditions and how this would impact assessments of its environmental toxicity, which currently only examines the diasteromers ephedrine and pseudoephedrine. Additionally, the paper explores an observation from literature that ephedrine isomers may interconvert to form different isomers.

The initial intention of chapter two was to investigate both the environmental persistence and toxicity of each isomer of ephedrine and its metabolite nor-ephedrine, using a combination of river water microcosms and EC_{50} toxicity tests kits. A series of follow-up single isomer microcosm experiment were then performed to investigate if ephedrine isomers could interconvert under environmental conditions, which has been hypothesised to occur during wastewater treatment. The paper concludes by using human liver microsomes to check if ephedrine interconversion could occur during human metabolism, whilst a retrospective analysis of river water was used to investigate if ephedrine could be formed from other sources, such as the metabolism of cathinones. An additional interpretation section is provided at the end of the chapter (page 149), which includes statistical information surround ephedrine degradation that was not provided in the paper but is usefully in assessing the significance of the observations reported in the paper.

Stereochemistry of ephedrine and its environmental significance: exposure and effects directed approach

Jack Rice, Kathryn Proctor, Luigi Lopardo, Sian Evans, Barbara Kasprzyk-Hordern[1]

Department of Chemistry, University of Bath, Bath BA2 7AY, UK

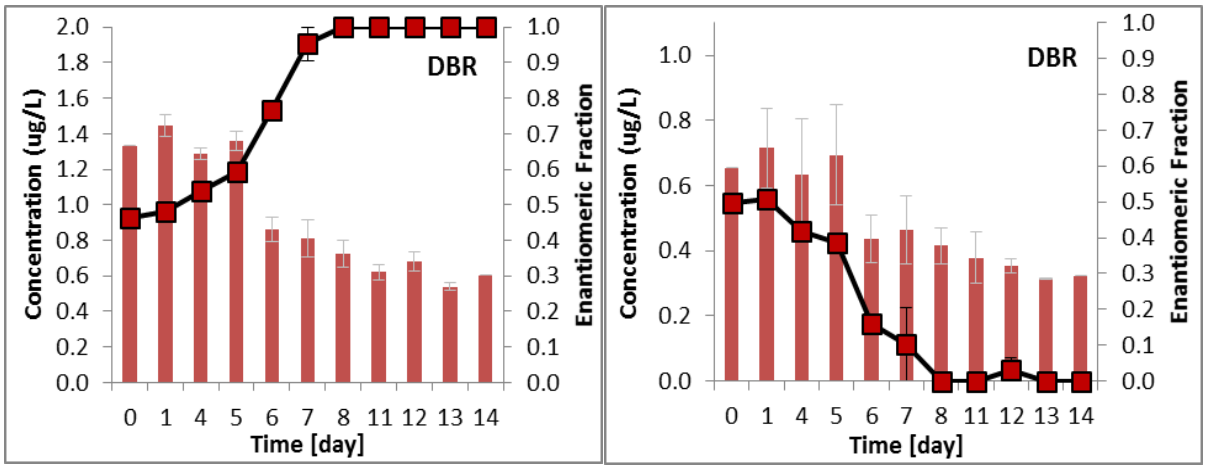
ABSTRACT

Analysis of drugs and pharmaceuticals in the environment is typically performed with non-chiral chromatographic techniques. The environmental risks posed by chiral compounds analysed in this way must therefore be assumed to be independent of chirality, meaning that each enantiomer is equally potent in toxicity and long-lived in stability. This manuscript examines the degradation of each of the four isomers of ephedrine in river simulating microcosms and links this to toxicity data obtained by exposing three different organisms (*D. magna, P. subcapitata and T. thermophila*) to each of the isomers individually. Microcosms showed that significant degradation only occurred in biotic conditions and that only two isomers (*1R,2S*-(-)-ephedrine, *1S,2S*-(+)-pseudoephedrine) degraded significantly over a period of fourteen days. This is concerning because at least one of the non-degraded isomers (*1S,2R*-(+)-ephedrine) has been observed in wastewater effluent, which discharges directly into rivers, meaning these isomers could be persistent in the environment. We also observed formation of *1S,2R*-(+)-ephedrine in single isomer *1R,2S*-(-)-ephedrine river simulating microcosms. Human liver microsome assays and mass spectrometry based data mining revealed that *1S,2R*-(+)-ephedrine is not human derived but it could be formed as a results of microbial metabolic processes. Across all three organisms tested the persistent isomers (*1S,2R*-(+)-ephedrine and *1R,2R*-(-)-pseudoephedrine) were more toxic than those that undergo degradation; meaning that if these isomers are entering or formed in the environment they might represent a potentially hazardous contaminant.

KEYWORDS: chiral, pharmaceuticals, wastewater, environment, river, enantiomeric profiling, enantiomeric fractions

[1] Corresponding author: E-mail: **b.kasprzyk-hordern@bath.ac.uk**; Fax: +44(0) 1225 386231; Tel: +44 (0) 1225 385013

Graphical abstract

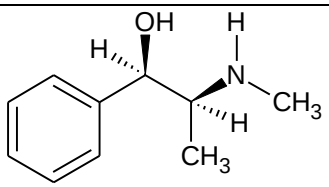
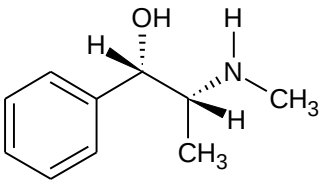
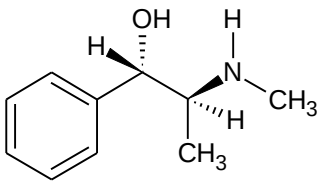


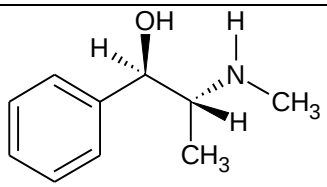
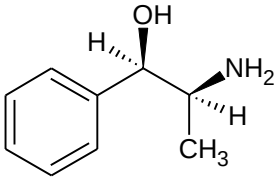
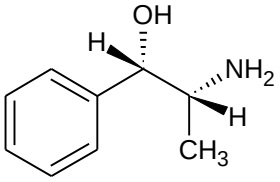
Stereoselective degradation of 1R,2S-(-)-ephedrine (left) and 1S,2S-(+)-pseudoephedrine (right) under biotic conditions in river simulating microcosms.

1. Introduction

Ephedrine has two chiral centres and can therefore exist in the form of four stereoisomers: *1R,2S*-(-)-ephedrine, *1S,2R*-(+)-ephedrine, *1S,2S*-(+)-pseudoephedrine and *1R,2R*-(-)-pseudoephedrine (Table 1). However, only two stereoisomers: *1R,2S*-(-)-ephedrine and *1S,2S*-(+)-pseudoephedrine are believed to exist in natural sources such as *ephedra*. *1R,2S*-(-)-ephedrine finds wide applications as a bronchodilator to treat bronchospasm associated with asthma, bronchitis and emphysema. It is also abused for its stimulant properties. *1S,2S*-(+)-pseudoephedrine is used as a decongestant [1].

Table 1. Studied chemicals and their properties

Compound	Structure	CAS No
1R,2S-(-)-ephedrine		299-42-3
1S,2R-(+)-ephedrine		24221-86-1
1S,2S-(+)-pseudoephedrine		345-78-8

1R,2R-(-)-pseudoephedrine		321-97-1
1R,2S-(-)-norephedrine		492-41-1
1S,2R-(+)-norephedrine		37577-28-9

Ephedrine has been detected previously in environmental matrices [2; 3] but with the usage of non-enantioselective methodology, which did not allow for stereoisomeric profiling of ephedrine. As a result this did not allow for an accurate assessment of the possible effects ephedrine might have on the environment. Stereoisomeric profiling is of vital importance as different stereoisomers of ephedrine differ significantly in potency, *1R,2S*-(-)-ephedrine has much higher stimulant properties than *1S,2S*-(+)-pseudoephedrine, and possibly also in toxicity to certain organisms.

In our previous study [1], a verification of the enantiomer-specific fate of ephedrine isomers was undertaken in a full scale WWTP and in receiving waters. Of the two enantiomers of (±)-ephedrine only the natural *1R,2S*-(-)-ephedrine enantiomer was frequently detected. However, 'non-natural' *1S,2R*-(+)-ephedrine was detected at low levels in only certain WWTPs throughout the sampling campaign, mainly in treated wastewater, which might suggest stereoselective processes occurring during treatment (e.g. chiral inversion although there is currently no experimental evidence to support this claim) leading to enrichment of ephedrine with *1S,2R*-(+)-enantiomer. It is worth noting that the most prevalent formation of *1S,2R*-(+)-ephedrine occurred during spring/summer time due to potentially higher activity of microorganisms. The possibility of chiral inversion occurring during treatment is of critical importance in understanding the fate of ephedrines in the environment and has to be studied further.

The verification of cumulative concentrations of ephedrines in raw wastewater indicated that higher levels of these compounds were observed during winter time (reaching 180 g/day in February in all studied WWTPs) than during summer time (< 80 g/day in August) [1; 4]. Interestingly, the analysis of diastereomeric fractions (DFs) of natural *1R,2S*-(-)-ephedrine and *1S,2S*-(+)-pseudoephedrine in raw wastewater revealed that over the winter months ephedrines were enriched with *1S,2S*-(+)-pseudoephedrine. This is possibly due to higher usage of over-the-counter medications (containing

1S,2S-(+)-pseudoephedrine) for the treatment of mild symptoms of cold. During the spring and summer months a reverse situation was observed as ephedrine was found to be enriched with a much more potent stimulant, *1R,2S*-(-)-ephedrine. This is a very important finding indicating that non-enantioselective measurement of ephedrines cannot be a reliable indicator of actual potency of ephedrines in the environment. Higher cumulative concentrations of ephedrines, which are enriched with less potent *1S,2S*-(+)-pseudoephedrine (as was observed during winter time in this study) might be of lower environmental significance than lower concentrations of ephedrines enriched with much more potent *1R,2S*-(-)-ephedrine (in summer in this study). Furthermore, wastewater treatment resulted in almost all cases in further enrichment of ephedrines in the aqueous phase with more potent *1R,2S*-(-)-ephedrine, with an average increase in DFs from 0.25 in raw wastewater to 0.35 in treated wastewater. Interestingly, the monitoring of receiving waters revealed that ephedrine was enriched with *1R,2S*-(-)-ephedrine at the beginning of the course of the river and its DFs decreased over the course of the river indicating an increase of *1S,2S*-(+)-pseudoephedrine (e.g. during the August sampling campaign DF of ephedrine denoted 0.93±0.03 at the beginning of the river course and decreased to 0.33±0.03 over 50 km downstream); a reverse situation to the one observed during wastewater treatment. This might indicate that different microbial communities are responsible for transformation of ephedrines during wastewater treatment and in the environment.

The absence of 1S,2R-(+)-ephedrine in influent wastewater suggests that it is not formed by metabolism of ephedrine in humans. However previous research into human metabolism of ephedrine was not carried out in a stereoselective manner and is mostly limited to 1R,2S-(-)-ephedrine, rather than the more prescribed and environmentally abundant 1S,2S-(+)-pseudoephedrine. Metabolic data indicates that 1R,2S-(-)-ephedrine is excreted primarily unchanged, with norephedrine and other metabolites forming in smaller quantities [5; 6]. Whilst literature data was not available for the metabolism of other ephedrine isomers in humans, metabolism of 1S,2R-(+)-ephedrine in rabbits and rabbit liver microsomes was observed to be slower than metabolism of 1R,2S-(-)-ephedrine [7]. Whilst this shows that a metabolic preference for the naturally occurring isomer, which may be important for the formation of synthetic isomers in the environment, rabbit metabolism of ephedrine is different from human metabolism with only a small percentage of (±)-ephedrine excreted [7; 8] or isolated from rabbit liver microsomes [7].

Our previous research raises several questions undermining validity of widely accepted environmental risk assessment procedures for pharmacologically active compounds. This includes lack of appreciation of the phenomenon of chirality in environmental risk assessment (ERA) for human and veterinary medicines [9; 10]. This paper attempts to answer the most urgent questions regarding the significance of stereochemistry of pharmaceuticals (using stereoisomers of ephedrine as a model example) in the context of their environmental fate and effects. To the authors' knowledge this is the

first report tackling stereoselective transformation of ephedrines in river simulating microcosms and associated enantiomer-specific ecotoxicity.

2. Experimental

2.1. Chemicals and materials

Reference standards: *1R,2S*-(-)-ephedrine [(-)-Eph], *1S,2R*-(+)-ephedrine [(+)-Eph], *1S,2S*-(+)-pseudoephedrine [(+)-Pse], *1R,2R*-(-)-pseudoephedrine [(-)-Pse], *1R,2S*-(-)-norephedrine [(-)-NorEph] and *1S,2R*-(+)-norephedrine [(+)-NorEph] were of ≥98% purity and were purchased from Sigma-Aldrich (Gillingham, UK). Surrogate/internal standards (SS/IS): 1S,2R-(+)-ephedrine-d3 (CAS No. 285979-73-9) and *R*/*S*-(±)-methamphetamine-d5 (CAS No. 60124-88-1, were purchased from Sigma Aldrich (Gillingham, UK) and Toronto Research Chemicals (Canada) respectively (Table 1). All surrogate/internal standards were added to the samples before extraction and were also used for the quantification of the analytes.

2.2. Sample preparation and analysis

Chiral drugs were extracted from surface water (50 mL) using Solid-Phase Extraction (SPE) and Oasis HLB cartridges (Waters, UK). All samples were spiked with 50 ng of each surrogate/internal standard and filtered with GF/F filters. Analytes were then eluted with 4 mL of MeOH and the extracts evaporated to dryness under nitrogen with a TurboVap evaporator (Caliper, UK, 40^{o}C, N_2, 5-15 psi) and reconstituted in 0.5 mL of mobile phase.

Waters ACQUITY UPLC^{TM} system (Waters, Manchester, UK) equipped with Chiral-CBH column, 100x2mm, 5µm (Chromtech, Congleton, UK) and Chiral-CBH 10x2.0mm guard column (Chromtech, Congleton, UK) were used for the analysis of enantiomers of ephedrines. The separation of ephedrines was undertaken using two different methods depending on the experiment. Samples from river simulating microsom experiments were analysed using the method described by Evans et al. [11] . The elution order of the four ephedrine isomers was: 1S,2R-(+)-ephedrine, 1R,2R-(-)-pseudoephedrine, 1R,2S-(-)-ephedrine, 1S,2S-(+)-pseudoephedrine. Samples from human liver microsome experiments were using the method described by Castrignanò et al. [12]. The elution order of the four ephedrine isomers was the same as in the previous method, but with co-elution of 1R,2S-(-)-ephedrine and 1R,2R-(-)-pseudoephedrine. This co-elution was not considered a hindrance for the analysis undertaken in the human liver microsome experiments. An injection volume of 20 µL was used in both experiments. Method validation parameters for both methods are summarised in Tables S1-4.

A XevoTQD (triple quadrupole) mass spectrometer (Waters, Manchester, UK) equipped with an electrospray ionisation source (ESI) was used for the quantification of ephedrines in both methods. The analyses were performed in ESI positive mode using multiple-reaction monitoring (MRM).

Nitrogen was used as the nebulising gas at a flow rate of 500 L/Hr, supplied by a high-purity nitrogen generator (Waters, Manchester, UK). Argon (99.998%) was used as the collision gas and supplied by BOC cylinder. MassLynx 4.1 (Waters, UK) and TargetLynx (Waters, UK) software was used to collect and analyse the obtained data.

The relative concentration of enantiomers of chiral drugs was expressed as the enantiomeric fraction (*EF*) and was calculated with the following equation:

$$Equation\ 1: EF = \frac{C_{(+)-enantiomer}}{C_{(+)-enantiomer} + C_{(-)-enantiomer}}$$

Where C-*(+)-enantiomer* and C-*(-)-enantiomer* are concentrations for the (+) and (-) enantiomers of (±)-ephedrine or (±)-pseudoephedrine. *EF* equals 1 or 0 in the case of single enantiomer form and 0.5 in the case of racemate.

A range of common inorganic ions (NO_2^-, NO_3^- and NH_4^+) and chemical oxygen demand (COD) were quantified to assess environmental conditions at the time of sampling and for experimental monitoring. Commercially available testing kits (purchased from Merck) were used and concentrations determined photochemically utilising a Merck Spectroquant® Pharo 300 spectrophotometer. Collected surface water was tested before spiking with ephedrine and again after a two week testing period.

2.3. River simulating microcosm experiments

2.3.1. Mixed river simulating microcosms

Mixed compound microcosm experiments were conducted to investigate the fate of ephedrines at an enantiomeric level due to biodegradation, photodegradation and other abiotic processes including sorption. River water for the microcosm bioreactor experiments was collected from a large river in the South-West of the UK in November.

Degradation experiments were conducted with and without light, to study photochemical and physical processes, e.g. hydrolysis and sorption, and with or without sodium azide, as an inhibitor to biotic processes (see Figure S1). Eight conical flasks, made of borosilicate 3.3 glass with no visible light absorption and UV light cut-off at <275 nm, were used as bioreactors in all microcosm experiments and were autoclaved prior to use. All were subsequently spiked with 1µg/L each of 1S,2R-(+)-ephedrine, 1R,2S-(-)-ephedrine, 1S,2S-(+)-pseudoephedrine, 1R,2R-(-)-pseudoephedrine, 1R,2S-(-)-norephedrine and 1S,2R-(+)-norephedrine in methanol, which was allowed to evaporate. Norephedrine was tested alongside ephedrines as it is the primary metabolite of ephedrine [5]. Microcosms were then filled with 2L of unfiltered river water and four were spiked with sodium azide to a concentration of 1g/L to inhibit biotic processes (Abiotic Reactors). Four bioreactors remained un-spiked in order to allow biotic processes to occur (Biotic Reactors). Two biotic and two abiotic

reactors were then wrapped in aluminium foil (Dark Reactors) and the remaining two biotic and two abiotic reactors left uncovered (Light Reactors). To limit outside contamination of the microcosms, all eight were plugged at the top with cotton wool, as this still allowed for the flow of air into the microcosm. Finally each reactor had a magnetic stirrer bar added and were placed onto unheated magnetic stirring pads at the lowest speed that allowed a vortex to form.

Daylight conditions were simulated using an Osram400W powerstar® HGI®-BT daylight lamp, which was switched on for eight hours each day to mimic average sunlight conditions in the UK. The bulb provides 23.64 μmol $S^{-1}m^{-2}$ per microamp of illumination at the 2L mark of a 2L conical flask, with the probe facing perpendicular to light source. The bulb provides 158.34 μmol $S^{-1}m^{-2}$ per microamp of illumination at the bottom of a dry 2L conical flask, with the probe directly facing the light source. All light intensity measurements were made using a LI-250A light meter with a quantum sensor. In order to decrease the effect of heat generated by the lamp all the microcosms, including those in the dark, were cooled using fans to ensure a roughly equal temperature inside each microcosm.

Samples were taken at regular intervals (once per day) over a fifteen-day sampling period and analysed as detailed by Evans et al. [11]. Other parameters analysed during the sampling period included dissolved oxygen (DO), pH, temperature, COD, ammonium, nitrate and nitrite (Figure S2).

2.3.2. Single-isomer ephedrine river simulating microcosms

Single ephedrine microcosms were carried out as an extension of the river water simulating microcosms described in 2.3.1, to examine the effects of chirality on ephedrine degradation. The microcosms were set up as described previously using river water collected from a large river in the South West of the UK in February and spiked with 1μg/L of either 1R,2S-(-)-ephedrine or 1S,2S-(+)-pseudoephedrine, the naturally occurring isomers. Eight microcosms were prepared in total (see Figure S3) four containing 1R,2S-(-)-ephedrine and four containing 1S,2S-(+)-pseudoephedrine. For each set of four single ephedrine microcosms two were wrapped in foil (Dark Reactors) and the others left exposed to eight hours a day of simulated daylight (Light Reactors) from an Osram400W powerstar® HGI®-BT daylight lamp as described previously.

Samples were taken at regular intervals (once per day) over a fifteen-day sampling period and analysed as detailed by Evans et al. [11]. Other parameters analysed during the sampling period included dissolved oxygen (DO), pH, temperature, COD, ammonium, nitrate and nitrite (Figure S4).

2.4. Human liver microsome metabolism

Human liver microsomes (HLMs) were set-up in accordance to the method described by Lopardo et al. [13] and were performed in duplicate for both 1R,2S-(-)-ephedrine and 1S,2S-(+)-pseudoephedrine, with each isomer examined in isolation. Metabolism studies were carried out to investigate if human metabolism of either ephedrine isomer lead to the formation of any other isomer. Currently available data on (±)-ephedrine metabolism in humans suggests that if metabolised ephedrine will primarily undergo hydroxylation or demethylation [5; 6]. The microsomes were incubated for a total of six hours as this matched the expected in vitro half-life of 1R,2S-(-)-ephedrine [5]. To determine absolute ephedrine and norephedrine isomer concentrations the samples were analysed as described by Castrignanò et al. [12].

2.5. Retrospective analysis with UHPLC-QTOF – screening for precursors of 1S,2R-(+)-ephedrine

River water samples collected during seven consecutive days in South-West England were analysed in accordance to the method described by Lopardo et al. [13]. Briefly, river water samples were filtered using GF/F glass microfibre filter 0.75 µm (Fisher Scientific, UK) followed by a solid phase extraction (SPE) using HLB Oasis® cartridges Water, UK) and concentrated 400-fold. Extracts were then dried under nitrogen using a TurboVap evaporator (Caliper, UK, 40◦C). Dry extract was then reconstituted in 250 µL 80:20 H_2O:MeOH, transferred to polypropylene vials.

A Dionex Ultimate 3000 HPLC (Thermo Fisher UK Ltd.) coupled with a Bruker Maxis HD Q-TOF (Bruker) equipped with an electrospray ionization source was utilized for the analysis of extracts. ESI positive and negative mode acquisition was performed in broadbandCID acquisition mode. HyStar™ Bruker was used to coordinate the LC-MS system. Chromatographic separation and MS source conditions are described by Lopardo et al. [13].

After analysis, data extracted from the Bruker system were processed with MetID software (Advanced Chemistry Development, Inc., ACD/Labs, UK) in order to predict metabolite structures.

2.6. Toxicity testing

2.6.1. Daphnia magna toxicity tests

The experiment was performed using Daphtoxkit F Magna (Laboratory for Biological Research in Aquatic Pollution, Ghent University, Belgium) in accordance with test procedures prescribed by national and international organizations (OECD test no. 202 [14]).

24h to 48h EC50 (or LC50) bioassays were performed in multiwell test plates starting from neonates, uniform in size and in age, hatched from ephippia. In order to provide the neonates hatched from the ephippia with food prior to the test, a 2h “pre-feeding” was applied with a suspension of Spirulina micro-algae. Each ephedrine isomer was tested individually at the following concentrations 7.8, 15.6,

31.3, 62.4, 125, 250, 500, 1000 mg/L. These were prepared by serial dilution of an initial 1g/L ephedrines solution with standard fresh water [14]. For a statistically acceptable evaluation of the effects each test concentration, as well as the control, were assayed in four replicates of five neonates. *Daphnia magna* neonates were incubated at 20-22^{o}C for 48 hours and the number of dead or immobilised organisms was counted after 24 and 48 hours. The EC_{50}/LC_{50} is the concentration where 50% of the Daphnia are dead or immobilised, determined by if they can swim freely after gently agitating the solution.

2.6.2. Pseudokirchneriella subcapitata toxicity tests

The experiment was performed using Algaltoxkit F (Laboratory for Biological Research in Aquatic Pollution, Ghent University, Belgium) in accordance with test procedures prescribed by national and international organizations (e.g. 'Algal growth inhibition test' (OECD Guideline 201 [15]) and the ISO "Water Quality - Freshwater Algal Growth Inhibition Tests with Unicellular Green Algae" (ISO Standard 8692). A 72h algal growth inhibition test was performed with Pseudokirchneriella subcapitata de-immobilized from algal beads. The test is based on the measurement of the optical density of algal cell suspensions (at 670 nm) in spectrophotometric cells of 10 cm path-length. Optical densities were then converted into algal numbers with the aid of the regression formula. The algae density was measured by photometry absorbance at 670 nm and it was diluted with culturing medium to achieve a density of 1.10^{6}. Each ephedrine isomer was individually diluted to a concentration of 500 mg L^{-1}, 300 mg L^{-1}, 160 mg L^{-1}, 50 mg L^{-1}, 5 mg L^{-1} and 0 mg L^{-1} with culturing medium. Algae were added to these dilutions to achieve a density of 1.10^{4} mL^{-1}. 25 mL of this solution was placed in 10 cm spectrophotometer cells, in triplicate. The absorbance was measured every 24 hours after agitation to re-suspend the algae. They were stored at 20 °C under cool white fluorescent lamps in a random order. The data was statistically analyzed using the Algaltox kit F Data treatment ErC50 spreadsheet.

2.6.3. Tetrahymena thermophila toxicity tests

The 24 h protozoan growth inhibition bioassay was performed using Protoxkit F (Laboratory for Biological Research in Aquatic Pollution, Ghent University, Belgium). *Tetrahymena thermophila* were chosen for toxicity testing due to their sensitivity to a variety of emerging organic contaminants at environmentally relevant concentrations, as well as their position within the ecosystem and the potential for further bioaccumulation [16].

The test was carried out in disposable spectrophotometric cells of 1 cm path-length to measure changes in optical density (OD) at 440nm. Each test cell contains *T. thermophila* ciliate inoculum (40uL), food substrate (40uL) and known concentration of the isomer or isomers being tested in synthetic freshwater (pH 7.75±0.19, 2mL). The OD measurements were taken at T0h and at T24h. At T0h the turbidity of the test cell will be high due to the food substrate. Over 24h the turbidity will

drastically decrease as the uninhibited growth of the ciliates will consume the food substrate. The degree of inhibition can be calculated from the difference in OD between the control cells and the test cells after 24 hours.

A preliminary investigation was carried out for 1R,2S-(-)-ephedrine and 1S,2S-(+)-pseudoephedrine to ascertain the approximate range between 100% inhibition and uninhibited growth across 4 orders of magnitude. Based on these results (see Supplementary Table 14 and 17), further definitive tests were carried out for each isomer between the lowest concentrations with a population growth inhibition of 80-100% and the highest concentration with an inhibition between 0-20%. To ensure the tests were valid each concentration was examined in duplicate and the control had to reach 60% OD decrease after 24h. Some test required an extra 2-4 hours to reach the validation criteria, which was batch dependent and indicated a slightly slower growth of the ciliates. The EC50 values for this study were calculated using 28h results.

3. Results and discussion

3.1. Stereoselective degradation of a mixture of ephedrine stereoisomers in river simulating microcosms

Degradation of a mixture of all four ephedrine stereoisomers and norephedrine enantiomers was studied in well-defined laboratory river water microcosm experiments. The following parameters were investigated: microbial degradation, photochemical reactions as well as other physicochemical processes such as sorption.

As can be observed from Figure 1 during a 14-day period (±)-ephedrine degradation only occurs in biotic microcosms, which indicates that microbial metabolic processes are the main degradation pathway for this compound in the environment (under studied experimental conditions). It is worth noting that the rate of biodegradation is higher in dark biotic microcosms than in those exposed to light. This shows that ephedrine is photostable under the experimental conditions. Furthermore, biotic degradation of ephedrine shows high stereoselectivity favouring degradation of natural 1R,2S-(-)-ephedrine and leading to enrichment of (±)-ephedrine with synthetic 1S,2R-(+)-enantiomer. This process is much more pronounced in dark biotic microcosms.

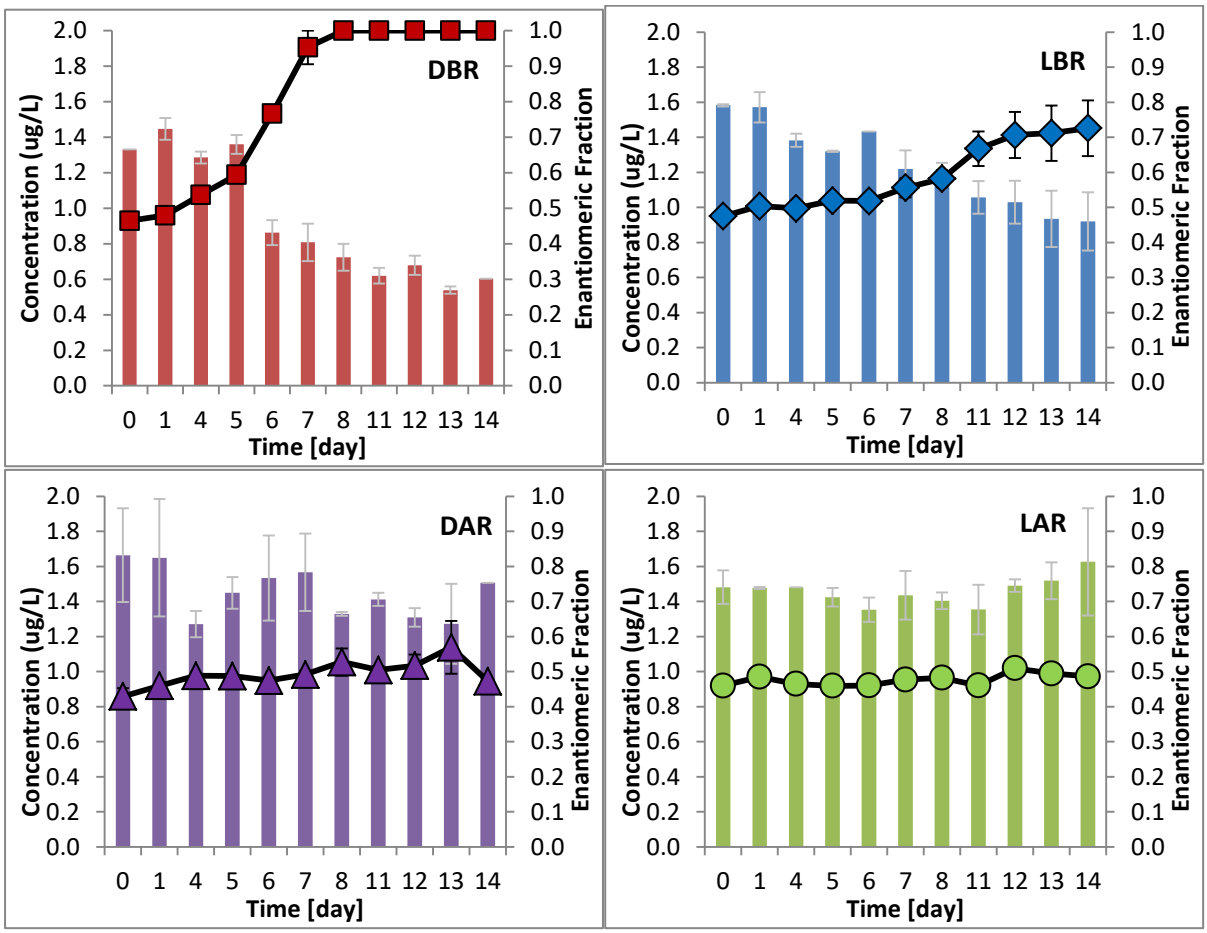


Figure 1. Mixed-compound river simulating microcosms – (±)-ephedrine degradation under dark abiotic (DAR), dark biotic (DBR), light abiotic (LAR) and light biotic (LBR) conditions (concentrations are represented by bars, enantiomeric fractions are represented by symbols). See tables S10-S13 for raw data.

Pseudoephedrine was found to degrade in a similar manner to ephedrine (Figure 2). It shows high photostability in both biotic and abiotic microcosms and no changes in enantiomeric composition were observed in both light and dark abiotic microcosms, whilst microbial metabolic processes are effective in the degradation of pseudoephedrine. These processes show high stereoselectivity with preferential degradation of 1S,2S-(+)-pseudoephedrine and subsequent enrichment of pseudoephedrine with synthetic 1R,2R-(-)-enantiomer. Similarly to ephedrine, degradation of pseudoephedrine is faster and shows higher stereoselectivity in the absence of external light. The increased rate of 1R,2S-(-)-ephedrine and 1S,2S-(+)-pseudoephedrine degradation in the biotic dark microcosms compared to biotic light might be due to the growth of algae in the presence of light and needs to be investigated further.

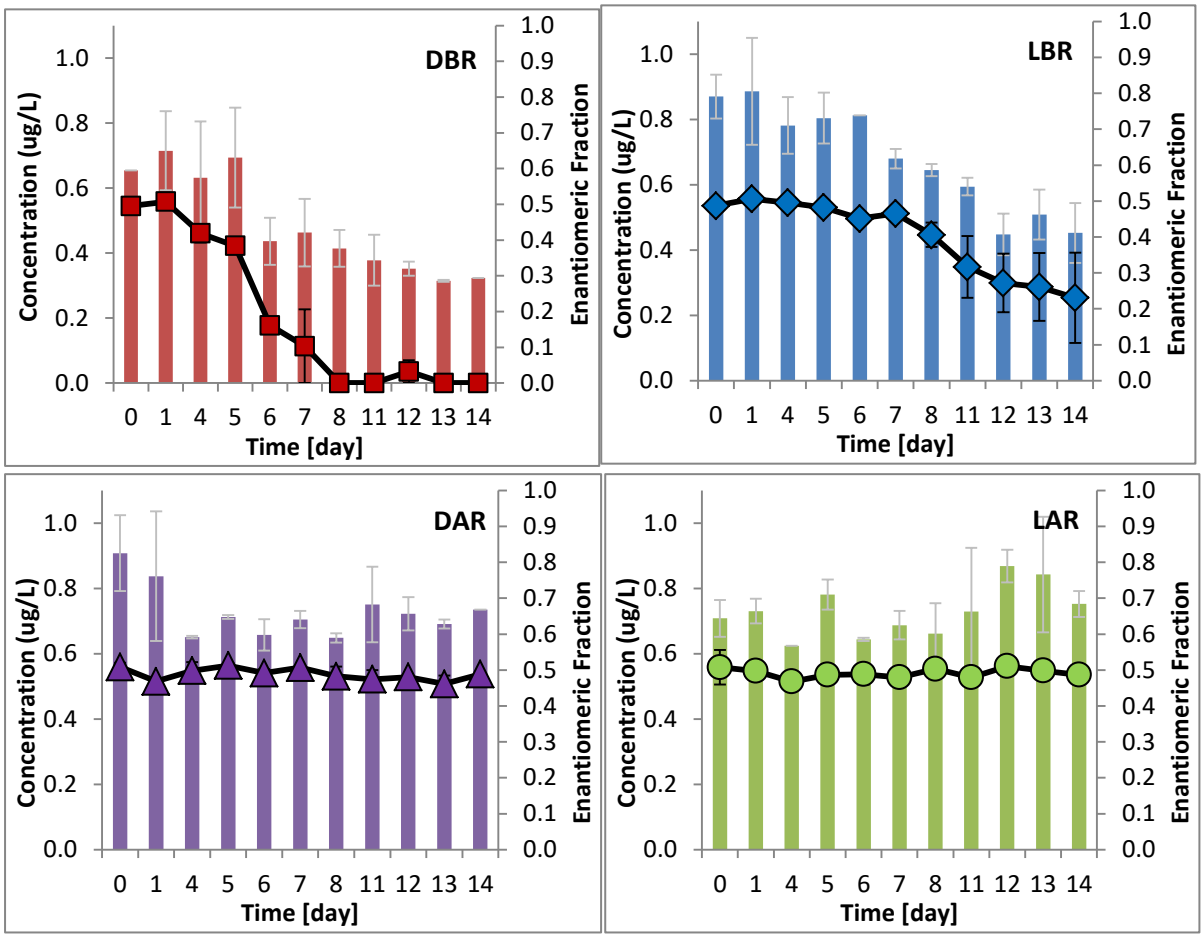


Figure 2. Mixed-compound river simulating microcosms – pseudoephedrine degradation under dark abiotic (DAR), dark biotic (DBR), light abiotic (LAR) and light biotic (LBR) conditions (concentrations are represented by bars, enantiomeric fractions are represented by symbols). See tables S10-S13 for raw data.

Similar to ephedrine and pseudoephedrine, degradation of norephedrine was observed in the biotic microcosms only indicating an importance of microbial metabolic processes (Figure 3). (±)-Norephedrine was examined in this experiment due to its potential importance as a breakdown product of (±)-ephedrine in animals and man [5-8], however significant (±)-norephedrine formation has not been observed in either of the biotic microcosms. Daily pH, temperature and dissolved oxygen (DO) measurements on each microcosms showed little variation between replicate samples (Figure S2), as well as limited inter-microcosm variation.

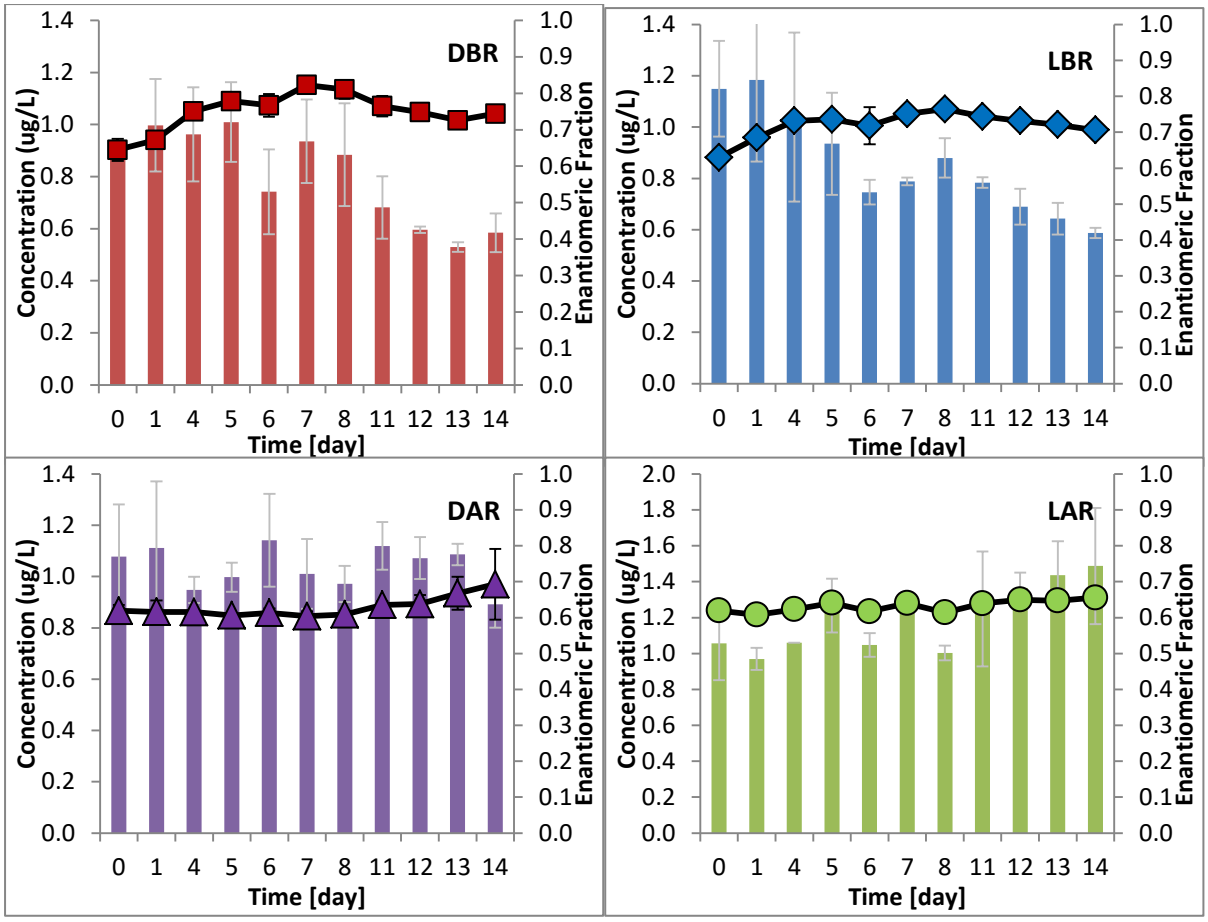


Figure 3. Mixed-compound river simulating microcosms – norephedrine degradation under dark abiotic (DAR), dark biotic (DBR), light abiotic (LAR) and light biotic (LBR) conditions (concentrations are represented by bars, enantiomeric fractions are represented by symbols). See tables S10-S13 for raw data.

3.2. Single 1R,2S-(-)-ephedrine or 1S,2S-(+)-pseudoephedrine river simulating microcosms

In the light of the observed extensive biodegradation of naturally occurring 1R,2S-(-)-ephedrine and 1S,2S-(+)-pseudoephedrine, single isomer 1R,2S-(-)-ephedrine or 1S,2S-(+)-pseudoephedrine river simulating microcosms were undertaken to verify if chiral inversion leading to the formation of non-natural 1S,2R-(+)-ephedrine or 1R,2R-(-)-pseudoephedrine can occur in the aqueous environment (Figure S3). Formation of 1S,2R-(+)-ephedrine was observed in a single-isomer biotic-light 1R,2S-(-)-ephedrine microcosm (Figure S5). It is important to note that the river water used in the microcosm experiments did not contain any ephedrine isomers prior to the start of the experiment. The observed peak passed method identification criteria, including retention time and MRM transition ratios, and is possibly formed as a result of chiral inversion, albeit after long residence time of 14 days and despite limited degradation of 1R,2S-(-)-ephedrine (Figure S6). Whilst not completely definitive this is a very important result, as it matches the observation of Kasprzyk-Hordern and Baker [1] showing formation of 1S,2R-(+)-ephedrine after biological wastewater treatment. The limited degradation of 1R,2S-(-)-ephedrine in this experiment (explained by matrix collection during heavy

rainfall and low biomass content) has hindered the observation of metabolite formation within early stages of the experiment.

3.3. Human liver microsome assays to verify human metabolism of 1R,2S-(-)-ephedrine and 1S,2S-(+)-pseudoephedrine

Kasprzyk-Hordern and Baker [1] observed formation of 1S,2R-(+)-ephedrine after biological wastewater treatment. Human liver microsomes assays were utilized to verify any human contribution to environmental occurrences of 1S,2R-(+)-ephedrine, via stereoselective metabolic processes of ephedrine isomers including chiral inversion.

Across the six hours of incubation a sample was taken and quenched at 1, 3 and 6 hours after addition of HLMs. At each of these time points there was no significant change in the concentration of 1R,2S-(-)-ephedrine relative to the control samples (Table S5), this is in line with literature data showing that 1R,2S-(-)-ephedrine is generally excreted unchanged in humans [5; 6]. This is further supported by the absence of (±)-norephedrine or any other ephedrine isomer, which were expected to be the main metabolite [5; 6] if 1R,2S-(-)-ephedrine were metabolised. The concentration of 1S,2S-(+)-pseudoephedrine however did decrease relative to the control sample, but no (±)-norephedrine or any other ephedrine isomers were observed. The HLM experiments for both compounds therefore supports the available literature data that ephedrines are mainly excreted un-metabolised and without chiral conversion. This also eliminates human metabolism of ephedrines as a source for the previous detection of 1S,2R-(+)-ephedrine in wastewater [1].

3.4. Retrospective analysis for 1S,2R-(+)-ephedrine precursors in river water

It is important to mention that there could be other sources of 1S,2R-(+)-ephedrine formation in water such as the reduction of R-(+)-methcathinone during human metabolism [17]. (±)-Methcathinone has been occasionally detected in wastewater influent [18; 19] but not in other environmental samples, and never with a focus on chirality. However as no ephedrine isomers were present in the river water used for microcosm experiments, and as relatively low percentages of methcathinone are excreted un-metabolised [17], it is postulated that 1S,2R-(+)-ephedrine was formed as a result of microbial transformation of 1R,2S-(-)-ephedrine. Further work is needed to confirm this hypothesis, however retrospective analysis of river water collected in a week long sampling campaign in England, using the procedure detailed in [13], showed no evidence of methcathinone.

3.5. Ecotoxicity of ephedrine stereoisomers to *Daphnia magna, Pseudokirchneriella subcapitata* and *Tetrahymena thermophila*

Ecotoxicity tests and obtained EC50 data for the non-natural isomers *1R,2R*-(-)-pseudoephedrine and *1S,2R*-(+)-ephedrine revealed that these isomers are more toxic to *Daphnia magna* than their naturally occurring enantiomers (Table 2). Their EC50s after 48h exposure were as follows: 107 and 171 mg/L in the case of *1R,2R*-(-)-pseudoephedrine and *1S,2R*-(+)-ephedrine respectively. $EC50_{48h}$ for the natural isomers *1R,2S*-(-)-ephedrine and *1S,2S*-(+)-pseudoephedrine, being widely used as prescription and over-the-counter medications, were much higher: 253 and 274 g/L respectively.

Table 2. Toxicity of ephedrine stereoisomers to *Daphnia magna, Pseudokirchneriella subcapitata* and *Tetrahymena thermophila*. Raw data is presented in figures S7-S11, tables S6-S9 and tables S14-S21.

	Daphnia magna		*Pseudokirchneriella subcapitata*	*Tetrahymena thermophila*
Single isomers	$EC50_{24h}$	$EC50_{48h}$	$EC50_{72h}$	$EC50_{24h}$
1S,2R-(+)-Ephedrine	373.1	170.8	754.5	42.6
1R,2S-(-)-Ephedrine	408.6	253.7	259.1	36.0
1S,2S-(+)-Pseudophedrine	528.3	274.3	417.9	99.3
1R,2R-(-)-Pseudophedrine	128.2	107.2	44.8	4.6
Mixtures				
Natural	-	-	-	61.8
All	-	-	-	52.4

Similarly, non-natural *1R,2R*-(-)-pseudoephedrine was found to be the most toxic isomer (<100 mg/L) for *Pseudokirchneriella subcapitata* after 72h exposure time. Interestingly, *1S,2R*-(+)-ephedrine was found to be less toxic than its natural isomer *1R,2S*-(-)-ephedrine and was found to be the least toxic isomer.

The results show *Tetrahymena thermophila* are far more sensitive to the presence of the ephedrine isomers than the other organisms explored in this study. As with the other organisms *1R,2R*-(-)-pseudoephedrine is by far the most toxic and its enantiomer *1S,2S*-(+)-pseudoephedrine the least: 4.6 and 99.3 mg/L, respectively. The ephedrine enantiomers *1S,2R*-(+)-ephedrine and *1R,2S*-(-)-ephedrine are much more similar in toxicity: 42.6 and 36.0 mg/L, respectively. As with *Pseudokirchneriella subcapitata* 1R,2S-(-)-ephedrine was more toxic than 1S,2R-(+)-ephedrine, although with less disparity.

The *Tetrahymena thermophila* $EC50_{24h}$ results for the ephedrine mixtures 'natural' (containing *1S,2S*-(+)-pseudoephedrine and *1R,2S*-(-)-ephedrine, DF = 0.5) and 'all' (which contains all the isomers, all EF and DF = 0.5) are 61.8 and 52.4 mg/L respectively. These two mixtures are less toxic than all of the individual isomers apart from *1S,2S*-(+)-pseudoephedrine, which is present in both mixtures, so it does not appear that there are any synergistic effects. All *Tetrahymena thermophila* $EC50_{24h}$ results are classified as harmful (< 100 mg/L), however *1R,2R*-(-)-pseudoephedrine is toxic (1-10 mg/L) according to the classification made by OECD and Commission of the European Communities [20-23]. For more detailed results please see Supplementary Tables 14-21.

4. Conclusion

To summarize, the high toxicity of *1S,2R*-(+)-ephedrine is of significant environmental importance. Kasprzyk-Hordern and Baker [1] have detected *1S,2R*-(+)-ephedrine in wastewater effluent, assumed to be formed during wastewater treatment, which is eventually discharged into the environment. HLM assays support previous literature evidence that human metabolism of ephedrines does not proceed with any conversion of chirality, so that 1S,2R-(+)-ephedrine formation is occurring after excretion or during wastewater treatment. Multi-compound microcosm experiments show that, if present, *1S,2R*-(+)-ephedrine is persistent within the environment, allowing it to become more widely spread. The detection of 1S,2R-(+)-ephedrine in single isomer microcosms containing only 1R,2S-(-)-ephedrine suggests that chiral inversion from biological processes is possible, further increasing the risk of pseudo-persistent environmental exposure to 1S,2R-(+)-ephedrine and that chiral inversion could occur during biological treatment of wastewater containing 1R,2S-(-)-ephedrine.

ACKNOWLEDGMENTS

Sian Evans and Jack Rice would like to acknowledge the University of Bath for funding their PhD studies. Kathryn Proctor would like to acknowledge NERC and Wessex Water for funding her CASE studentship (NE/L009579/1). Support from EPSRC (Project No. EP/I038608/1), NERC (Project No. NE/I000534/1, Project No. NE/L009579/1) and the Leverhulme Trust (Project No RPG-2013-297) is also acknowledged. All data supporting this study are provided as supporting information accompanying this paper.

SUPPORTING INFORMATION

Additional information containing details on materials used is available free of charge via the Internet

REFERENCES

[1] Kasprzyk-Hordern, B. and Baker, D.R., 2012. Enantiomeric Profiling of Chiral Drugs in Wastewater and Receiving Waters. *Environmental Science & Technology,* 46(3), pp. 1681-1691.
[2] Chiaia, A.C., Banta-Green, C. and Field, J., 2008. Eliminating solid phase extraction with large-volume injection LC/MS/MS: analysis of illicit and legal drugs and human urine indicators in U.S. wastewaters. *Environ Sci Technol,* 42(23), pp. 8841-8848.
[3] Postigo, C., de Alda, M.J.L. and Barcelo, D., 2008. Fully automated determination in the low nanogram per liter level of different classes of drugs of abuse in sewage water by on-line solid-phase extraction-liquid chromatography-electrospray-tandem mass spectrometry. *Analytical Chemistry,* 80(9), pp. 3123-3134.
[4] Kasprzyk-Hordern, B. and Baker, D.R., 2012. Estimation of community-wide drugs use via stereoselective profiling of sewage. *Science of the Total Environment,* 423, pp. 142-150.
[5] Wilkinson.Gr and Beckett, A.H., 1968. ABSORPTION METABOLISM AND EXCRETION OF EPHEDRINES IN MAN .2. PHARMACOKINETICS. *Journal of Pharmaceutical Sciences,* 57(11), pp. 1933-&.
[6] Sever, P.S., Dring, L.G. and Williams, R.T., 1975. METABOLISM OF (-)-EPHEDRINE IN MAN. *European Journal of Clinical Pharmacology,* 9(2-3), pp. 193-198.
[7] Feller, D.R. and Malspeis, L., 1977. BIOTRANSFORMATION OF D(-)-EPHEDRINE AND L(+)-EPHEDRINE IN RABBIT, INVIVO AND INVITRO. *Drug Metabolism and Disposition,* 5(1), pp. 37-46.
[8] Axelrod, J., 1953. STUDIES ON SYMPATHOMIMETIC AMINES .1. THE BIOTRANSFORMATION AND PHYSIOLOGICAL DISPOSITION OF L-EPHEDRINE AND L-NOREPHEDRINE. *Journal of Pharmacology and Experimental Therapeutics,* 109(1), pp. 62-73.
[9] EMEA, **Guideline on the ERA of medicinal products for human use;** European Medicines Agency, Doc. Ref. MEA/CHMP/SWP/4447/00Guideline on the ERA of medicinal products for human use; European Medicines Agency, Doc. Ref. MEA/CHMP/SWP/4447/00.
[10] EEC, ERA for Veterinary Medicinal Products other then GMO containing and Immunological Products; EU Directive 81/852/EEC.
[11] Evans, S.E., Davies, P., Lubben, A., and Kasprzyk-Hordern, B., 2015. Determination of chiral pharmaceuticals and illicit drugs in wastewater and sludge using microwave assisted extraction, solid-phase extraction and chiral liquid chromatography coupled with tandem mass spectrometry. *Analytica Chimica Acta,* 882, pp. 112-126.
[12] Castrignano, E., Lubben, A. and Kasprzyk-Hordern, B., 2016. Enantiomeric profiling of chiral drug biomarkers in wastewater with the usage of chiral liquid chromatography coupled with tandem mass spectrometry. *Journal of Chromatography A,* 1438, pp. 84-99.
[13] Lopardo, L., Cummins, A., Rydevik, A. and Kasprzyk-Hordern, B., 2017. New Analytical Framework for Verification of Biomarkers of Exposure to Chemicals Combining Human Biomonitoring and Water Fingerprinting. *Anal Chem*.
[14] OECD, 2004. *Test No. 202: Daphnia sp. Acute Immobilisation Test.* OECD Publishing.
[15] OECD, 2011. *Test No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test.* OECD Publishing.
[16] Gerhardt, A., Ud-Daula, A. and Schramm, K.W., 2010. Tetrahymena spp. (Protista, Ciliophora) as Test Species in Rapid Multi-level Ecotoxicity Tests. *Acta Protozoologica,* 49(4), pp. 271-280.
[17] Paul, B.D. and Cole, K.A., 2001. Cathinone (Khat) and methcathinone (CAT) in urine specimens: A gas chromatographic-mass spectrometric detection procedure. *Journal of Analytical Toxicology,* 25(7), pp. 525-530.
[18] Gonzalez-Marino, I., Gracia-Lor, E., Rousis, N.I., Castrignano, E., Thomas, K.V., Quintana, J.B., Kasprzyk-Hordern, B., Zuccato, E. and Castiglioni, S., 2016. Wastewater-Based Epidemiology To Monitor Synthetic Cathinones Use in Different European Countries. *Environmental Science & Technology,* 50(18), pp. 10089-10096.

[19] Mwenesongole, E.M., Gautam, L., Hall, S.W., Waterhouse, J.W. and Cole, M.D., 2013. Simultaneous detection of controlled substances in waste water. *Analytical Methods,* 5(13), pp. 3248-3254.
[20] OECD, 2001. Harmonised Integrated Classification System for Human Health and Environmental Hazards of Chemical Substances and Mixtures.: OECD publishing.
[21] Institute for health and consumer protection, 2003. *Technical guidance document on risk assessment in support of Commission Directive 93/67/ EEC on risk assessment for new notified substances and Commission Regulation (EC) No. 1488/94 on risk assessment for existing substances.*: European Chemicals Bureau.
[22] Petrie, B., Barden, R. and Kasprzyk-Hordern, B., 2015. A review on emerging contaminants in wastewaters and the environment: Current knowledge, understudied areas and recommendations for future monitoring. *Water Research,* 72, pp. 3-27.
[23] Cleuvers, M., 2005. Initial risk assessment for three beta-blockers found in the aquatic environment. *Chemosphere,* 59(2), pp. 199-205.
[24] Becker, D.E., 2012. Basic and clinical pharmacology of autonomic drugs. *Anaesthesia Progress,* 59(4), pp. 159-169.
[25] Kobayashi, S., Endou, M., Sakuraya, F., Matsuda, N., Zhang, X.H., Azuma, M., Echigo, N., Kemmotsu, O., Hattori, Y. and Gando, S., 2003. The sympathomimetic actions of l-ephedrine and d-pseudoephedrine: Direct receptor activation or norepinephrine release? *Anesthesia and Analgesia,* 97(5), pp. 1239-1245.
[26] Leclercq, M., Mathieu, O., Gomez, E., Casellas, C., Fenet, H. and Hillaire-Buys, D., 2009. Presence and Fate of Carbamazepine, Oxcarbazepine, and Seven of Their Metabolites at Wastewater Treatment Plants. *Archives of Environmental Contamination and Toxicology,* 56(3), pp. 408-415.
[27] Evans, S., Bagnall, J. and Kasprzyk-Hordern, B., 2017. Enantiomeric profiling of a chemically diverse mixture of chiral pharmaceuticals in urban water. *Environmental Pollution,* 230, pp. 368-377.
[28] US EPA. E.p. agency, 2007. *The risk quotient method and levels of concern.*

Supplementary information

Table S1. Optimised MRM conditions for the analysis of ephedrines by LC/MS/MS by Evans et al. (CV-cone voltage [V]; CE-collision energy [eV]).

Analyte	CV/CE	MRM1 (quantification)	CV/CE	MRM2 (confirmation)
1R,2S-(-)/*1S,2R*-(+)-Ephedrine	23/12	166.1 > 148.1	23/21	166.1 > 133.1
1S,2S-(+)/*1R,2R*-(-)-Pseudophedrine				
1R,2S-(-)/*1S,2R*-(+)-Norephedrine	23/10	152.2 > 134.1	23/16	152.2 > 117.1
R/S-(±)-Methamphetamine-d5	24/11	155.0 > 121.0	-	-
R/S-(±)-ephedrine-d3	23/12	169.2 > 148.1	23/21	169.2 > 133.1

Table S2. Method performance data for the method described by Evans et al.

Analytes	SS/IS t_r	t_r (min)[a]	Linearity range (µg L^{-1})	R^{2b}	MDL_{calc}[c] (µg L^{-1})	MDL_{calc}[d] (µg L^{-1})	R_s ± RSD[e]	EF ± RSD[f]	Rec ± %RSD (%)[g]	Inter-day precision
1S,2R(+)-Ephedrine	1.2 ± 0.0	24.13 ±0.51	0.375-250	0.997	1.83	6.11	1.8 ± 0.4	0.50 ± 0.12	120.20 ± 21.00	2.8
1R,2S(-)-Ephedrine	1.1 ± 0.0	21.23 ±0.53	0.025-250	0.997	0.97	3.24			94.80 ± 6.90	3.8
1S,2S(+)-Pseudophedrine	1.0 ± 0.0	27.62 ±0.53	0.050-250	0.997	1.46	4.85	2.8 ± 0.8	0.49 ± 0.05	56.60 ± 3.83	5.3
1R,2R(-)-Pseudophedrine	1.1 ± 0.0	22.30 ±0.29	0.500-250	0.997	1.38	4.60			76.85 ± 10.20	4.3
1R,2S(-)-Norephedrine	1.2 ± 0.0	21.81 ±0.49	0.025-250	0.997	1.64	5.47	0.9 ± 0.1	0.54 ± 0.09	86.30 ± 8.70	3.3
1S,2R(+)-Norephedrine	1.2 ± 0.0	23.50 ±0.49	0.025-250	0.997	1.63	5.42			74.40 ± 33.20	5.4

[a] *t_r - retention time*
[b] *R^2 - correlation coefficient*
[c] *MDL_{calc} - calculated method detection limit*
[d] *MQL_{calc} - calculated method quantification limit*
[e] *R_s - chromatographic resolution of enantiomers*
[f] *EF - enantiomeric fraction*
[g] *Rec - mean method (SPE) recovery (calculated for river water spiked with 50 and 250 µg L^{-1})*

Table S3. Optimised MRM conditions for the analysis of ephedrines by LC-MS/MS by Castrignanò et al.

Analyte	CV/CE	MRM1 (quantification)	CV/CE	MRM2 (confirmation)
1R,2S-(-)/*1S,2R*-(+)-Ephedrine	23/12	166.1 > 148.1	23/21	166.1 > 133.1
1S,2S-(+)/*1R,2R*-(-)-Pseudophedrine				
1R,2S-(-)/*1S,2R*-(+)-Norephedrine	23/10	152.2 > 134.1	23/16	152.2 > 117.1
R/S-(±)-ephedrine-d3	23/18	169.2 > 151.0	-	-

Table S4. Method performance data for the method described by Castrignanò et al.

Analytes	SS/IS t_r	t_r (min)[a]	Linearity range (µg L^{-1})	R^{2b}	MDL_{calc}[c] (µg L^{-1})	MDL_{calc}[d] (µg L^{-1})	R_s ± RSD[e]	EF ± RSD[f]	Rec ± %RSD (%)[g]	Inter-day precision %RSD
1S,2R-(+)-Ephedrine	0.6	12.3 ± 0.3	1.000-500	0.9974	0.0059	0.0295	0.9 ± 0.1	0.46 ± 0.10	84.67 ± 4.57	6.77 ± 3.73
1R,2S-(-)-Ephedrine & 1R,2R-(-)-Pseudoephedrine	0.5	13.4 ± 0.	0.500-500	0.9975	0.0024	0.0048	2.2 ± 0.9	0.46 ± 0.02	104 ± 4.23	5.13 ± 4.27
1S,2S-(+)-Pseudophedrine	1.9	32.94 ± 0.8	1.000-500	0.9903	0.0056	0.0280			89.33 ± 3.97	3.83 ± 1.67
E1-Norephedrine	0.4	13.6 ± 0.3	0.125-500	0.9981	0.0006	0.0011	0.9 ± 0.1	0.48 ± 0.02	112.33 ± 1.8	4.47 ± 0.53
E2-Norephedrine	2.2	15.1 ± 0.4	0.125-500	0.9983	0.0006	0.0012			97.67 ± 3.13	4.13 ± 1.37

[a] *t_r - retention time*
[b] *R^2 - correlation coefficient*
[c] *MDL_{calc} - calculated method detection limit*
[d] *MQL_{calc} - calculated method quantification limit*
[e] *R_s - chromatographic resolution of enantiomers*
[f] *EF - enantiomeric fraction*
[g] *Rec - mean method (SPE) recovery (calculated for river water spiked with 25, 250 and 2500 µg L^{-1})*

Table S5. Human liver microsome experiment showing ephedrine concentrations over time. Analyte blanks contain no microsomes, whilst HLM blanks contain microsomes but no analyte.

HLM experiment	(1R,2S)-(-)-ephedrine (µg L^{-1})	(1S,2S)-(+)-pseudoephedrine (µg L^{-1})
Time 0 Analyte blank	25.25 ± 0.45	35.40 ± 0.40
Time 1 Digest 1	33.90 ± 0.00	224.50 ± 2.60
Time 1 Digest 2	38.45 ± 0.45	178.15 ± 0.35
Time 1 Analyte blank	37.2 ± 0.20	159.40 ± 2.00
Time 3 Digest 1	33.00 ± 0.60	88.45 ± 1.25
Time 3 Digest 2	36.45 ± 0.35	74.30 ± 0.20
Time 3 Analyte blank	35.15 ± 0.85	88.15 ± 0.85
Time 6 Digest 1	28.75 ± 0.65	133.50 ± 3.10
Time 6 Digest 2	32.00 ± 0.00	104.55 ± 0.75
Time 6 Analyte blank	29.15 ± 0.35	143.70 ± 0.40
Time 6 HLM blank	<LOD, <LOD	15.00 ± 0.00
% degradation	(1R,2S)-(-)-ephedrine	(1S,2S)-(+)-pseudoephedrine
Time 1 % degradation	97.24 ± 1.66	126.30 ± 3.30
Time 3 % degradation	98.79 ± 5.13	92.31 ± 2.44
Time 6 % degradation	104.20 ± 3.61	82.83 ± 2.75

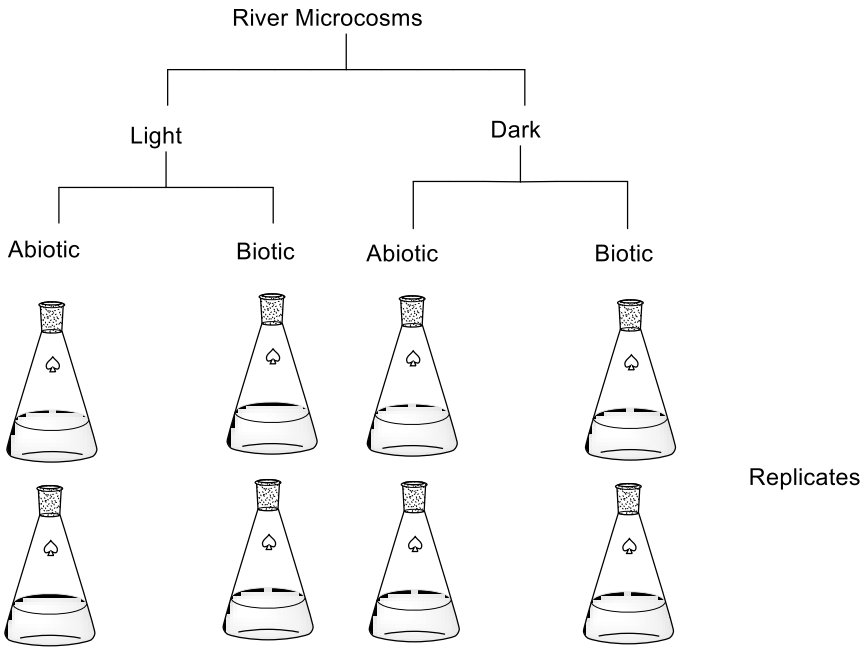
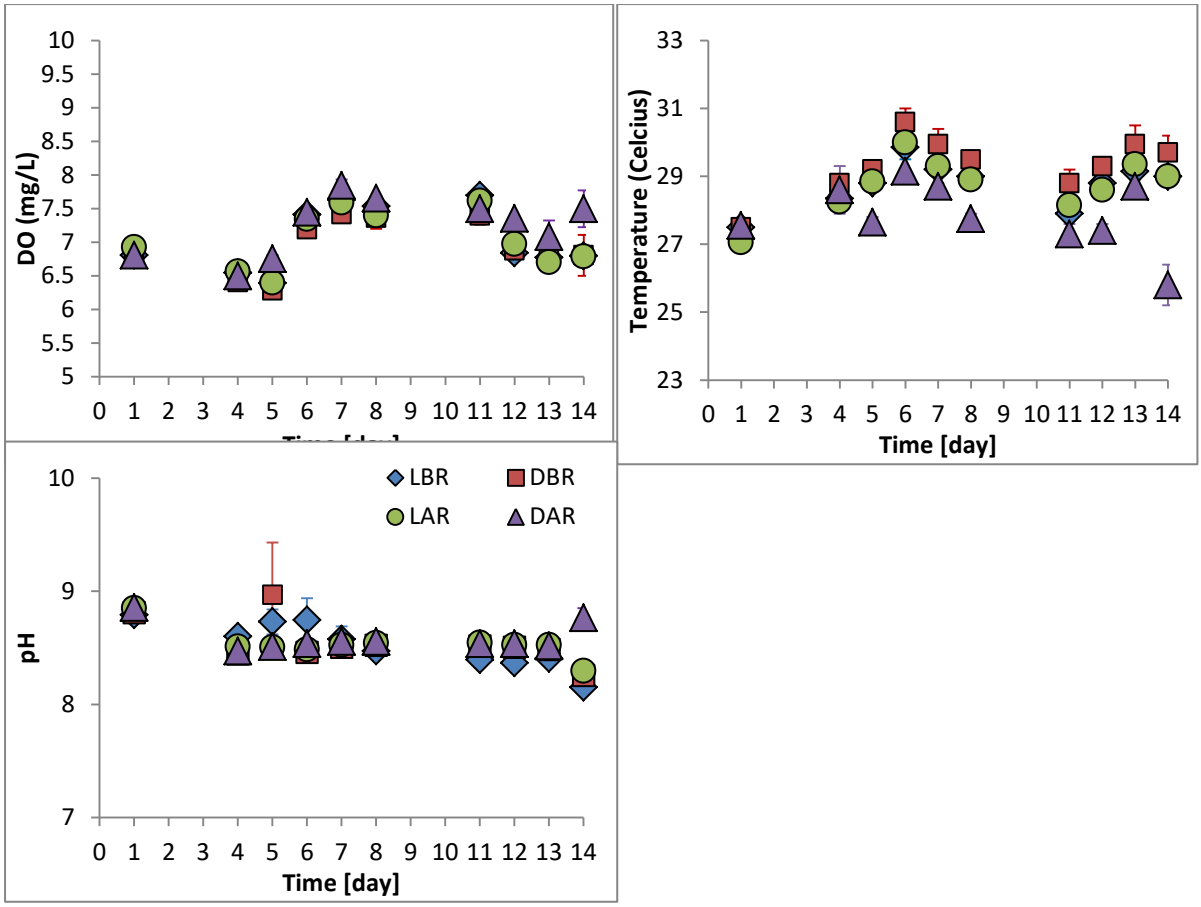


Figure S1. Summary of river simulating microcosms



Day	Microcosm	NH_4^+ mg/L	NO_3^- mg/L	NO_2^- mg/L	COD mg/L O_2
0	River water	No result	No result	No result	No result
14	Dark Abiotic	No result	No result	0.7	176
	Dark Biotic	No result	No result	1.9	< 25
	Light Abiotic	No result	No result	1.8	213
	Light Biotic	No result	No result	3.2	< 25

Figure S2. Mixed-compound river microcosms - temperature, dissolved oxygen (DO), pH, ammonium, nitrate, nitrite and chemical oxygen demand (COD)

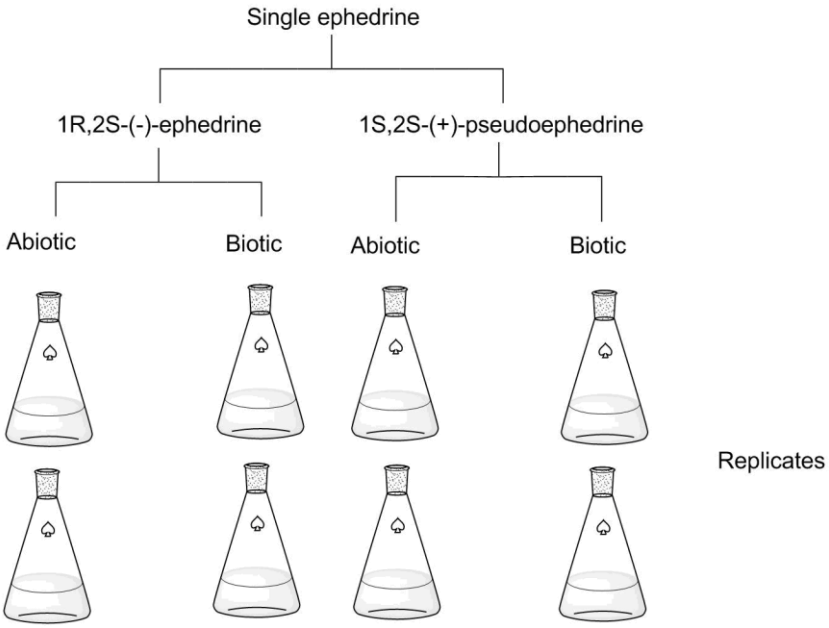
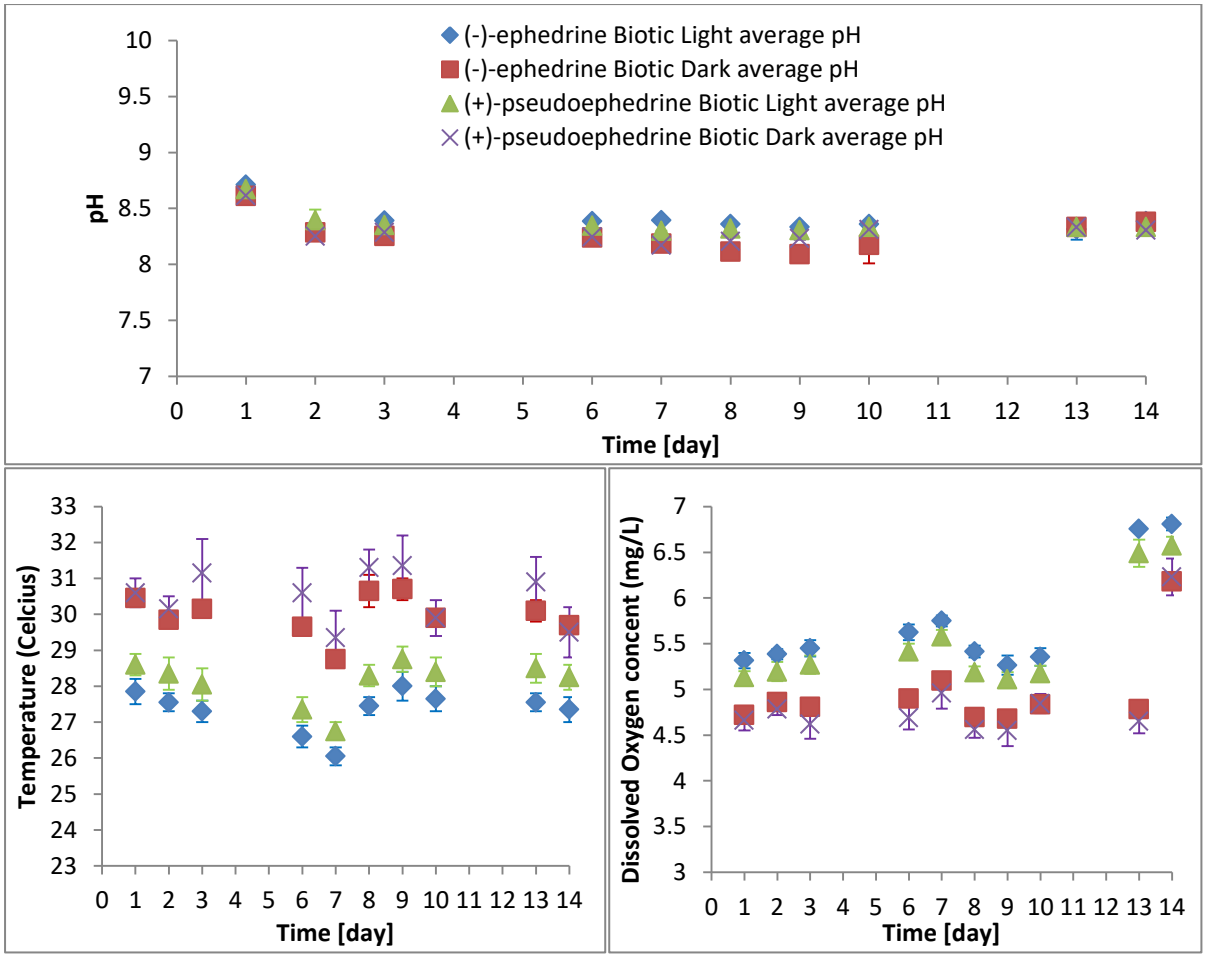


Figure S3. Summary of single-compound river microcosms



Day	Microcosm	NH_4^+ mg/L	NO_3^- mg/L	NO_2^- mg/L	COD mg/L O_2
0	River water	No result	No result	2.1	625
14	1R,2S-(-)-ephedrine Biotic Light	No result	No result	No result	90
	1R,2S-(-)-ephedrine Biotic Dark	No result	No result	No result	81
	1S,2S-(+)-pseudoephedrine Biotic Light	No result	No result	No result	117
	1S,2S-(+)-pseudoephedrine Biotic Dark	No result	No result	No result	344

Figure S4. Single-compound river microcosms - temperature, dissolved oxygen (DO), pH, ammonium, nitrate, nitrite and chemical oxygen demand (COD).

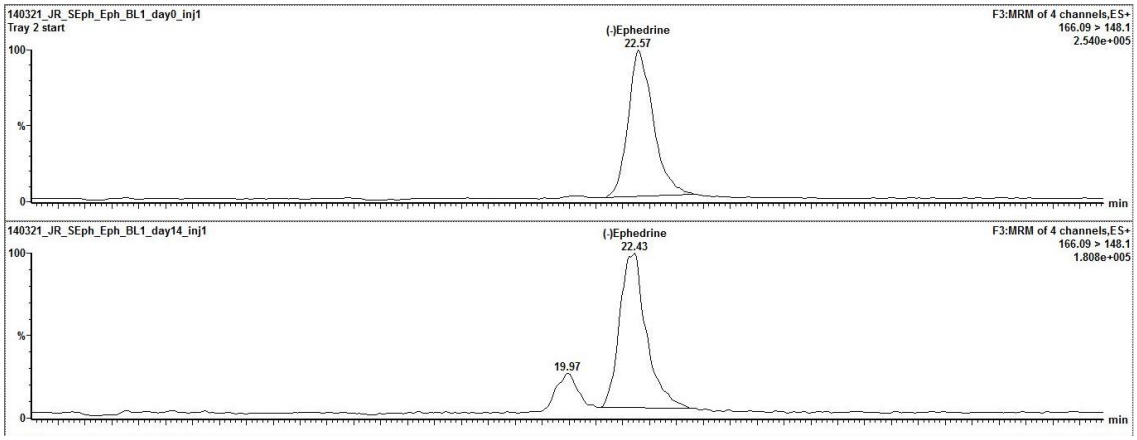


Figure S5. Formation of (+)-(1S,2R)-ephedrine (retention time, 19.97 minutes) in (-)-(1R,2S)-ephedrine river simulating microcosm (retention time, 22.43 minutes)

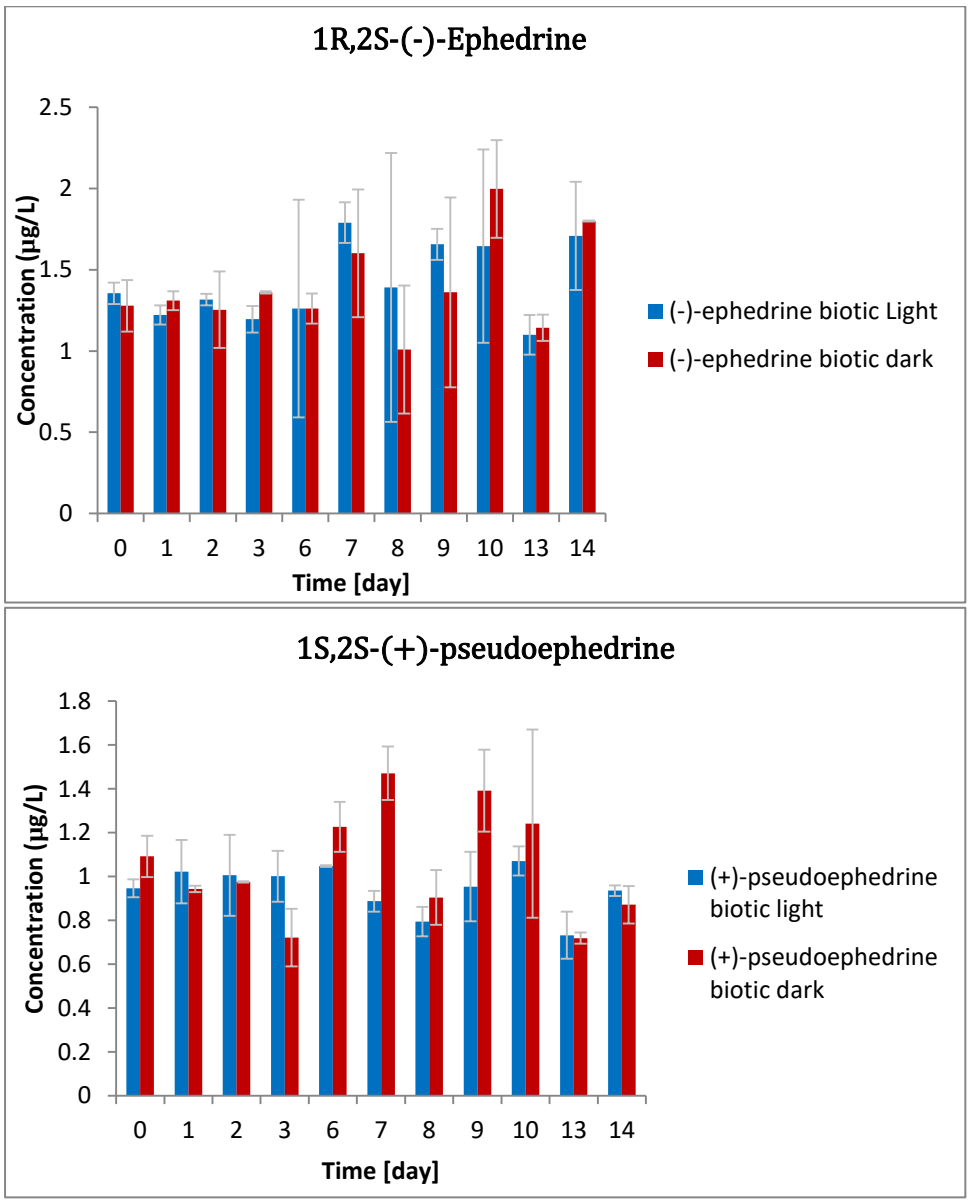
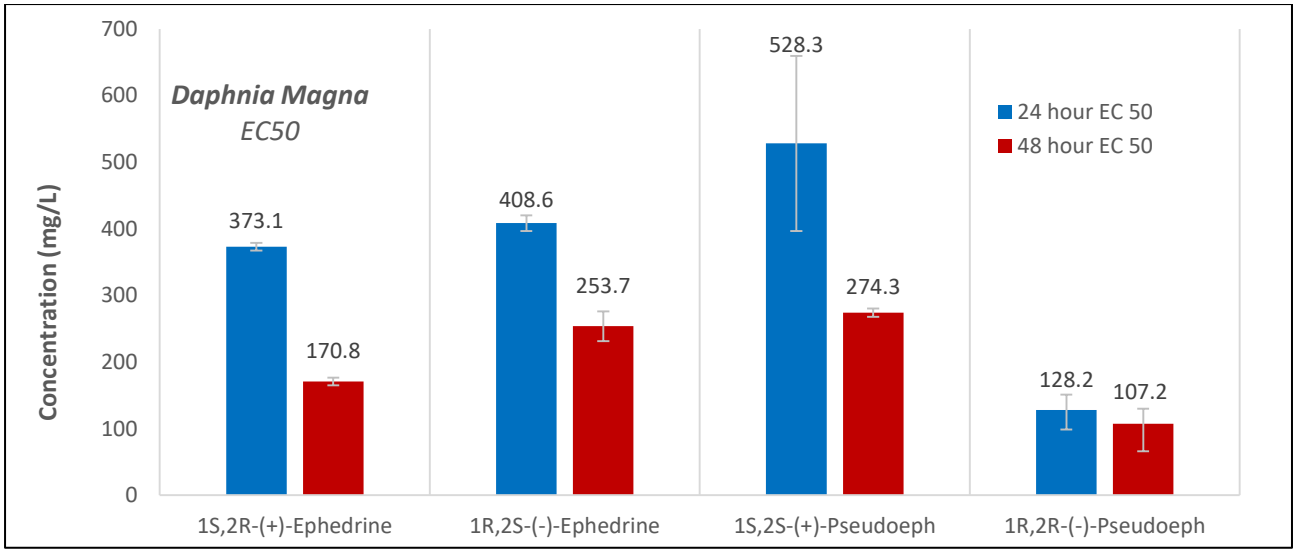
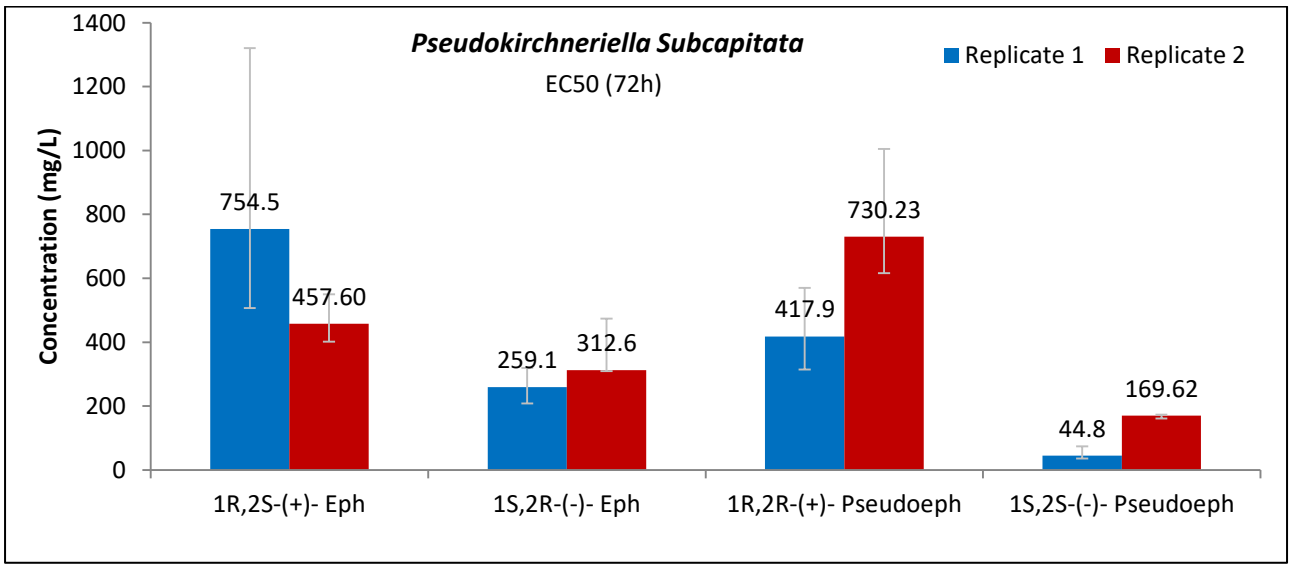


Figure S6. Single-compound river simulating microcosms – 1R,2S-(-)-ephedrine and 1S,2S-(+)-pseudoephedrine degradation under dark biotic (DBR) and light biotic (LBR) conditions





Note: *Replicate 2 for 1S,2S-(+)Pseudoephedrine in Selenastrum capricoruntum tests did not achieve the minimum growth inhibition required to validate the test, and therefore should be treated semi-quantitative.*

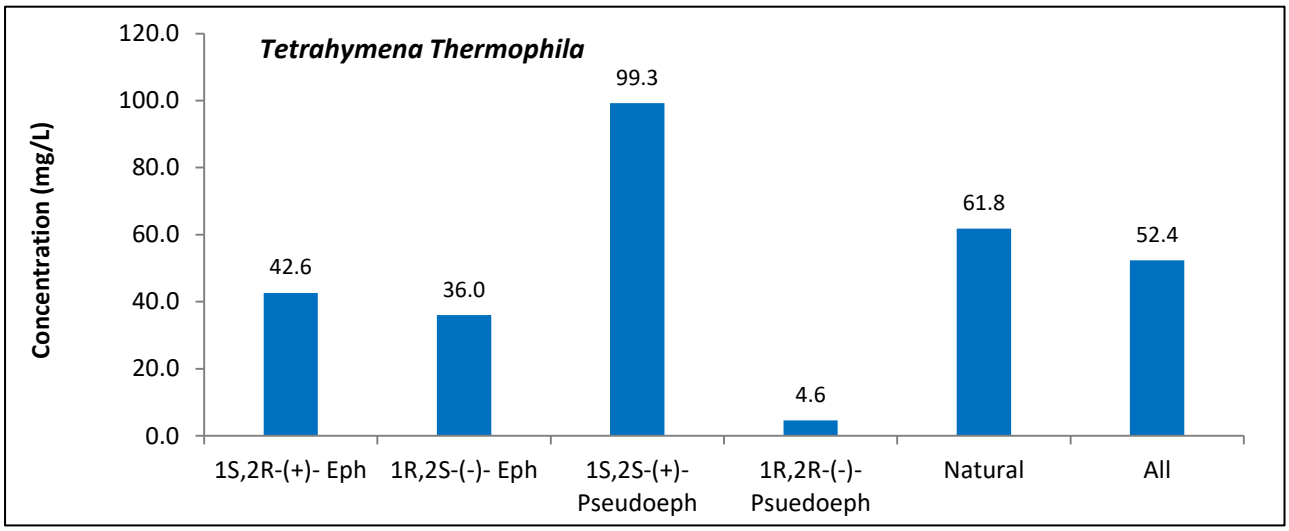


Figure S7 Toxicity of ephedrine stereoisomers to *Daphnia magna*, *Selenastrum capricornutum* and *Tetrahymena thermophila*. See tables S14-S21 for CV% of individual tests of *Tetrahymena thermophila*.

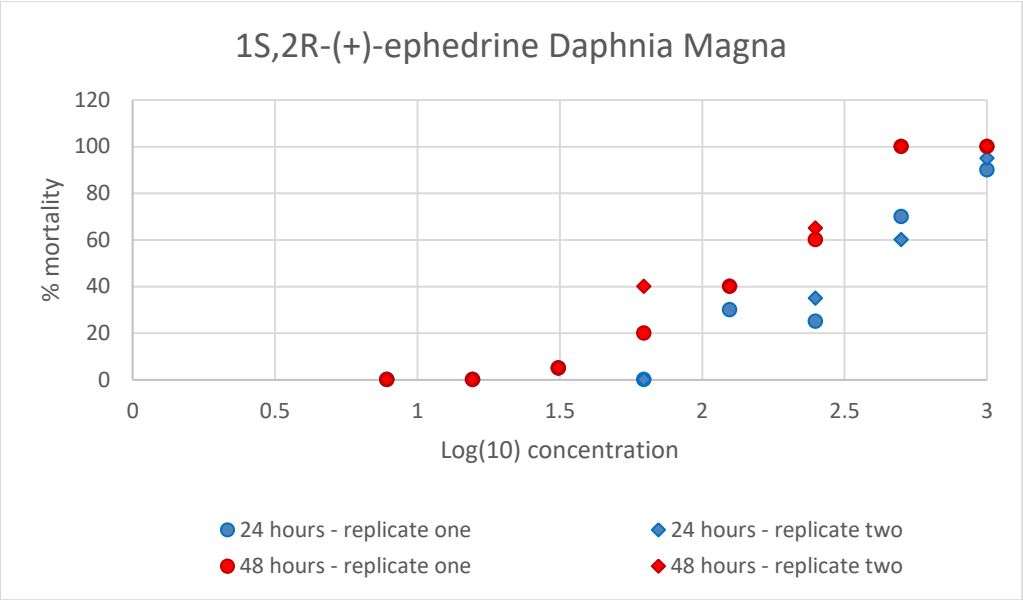


Figure S8. % mortality of *Daphnia Magna* exposed to 1S,2R-(+)-ephedrine

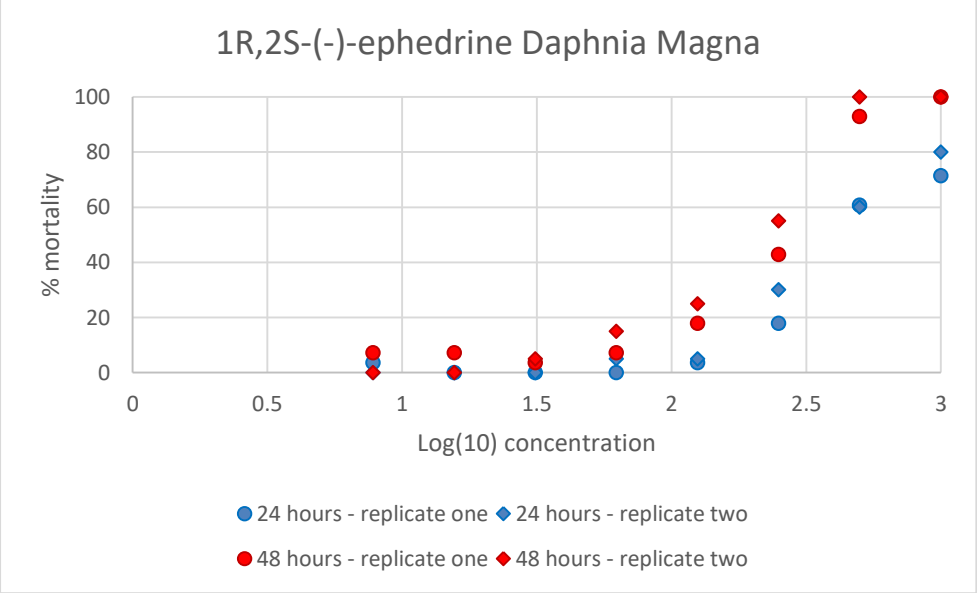


Figure S9. % mortality of *Daphnia Magna* exposed 1R,2S-(-)-ephedrine

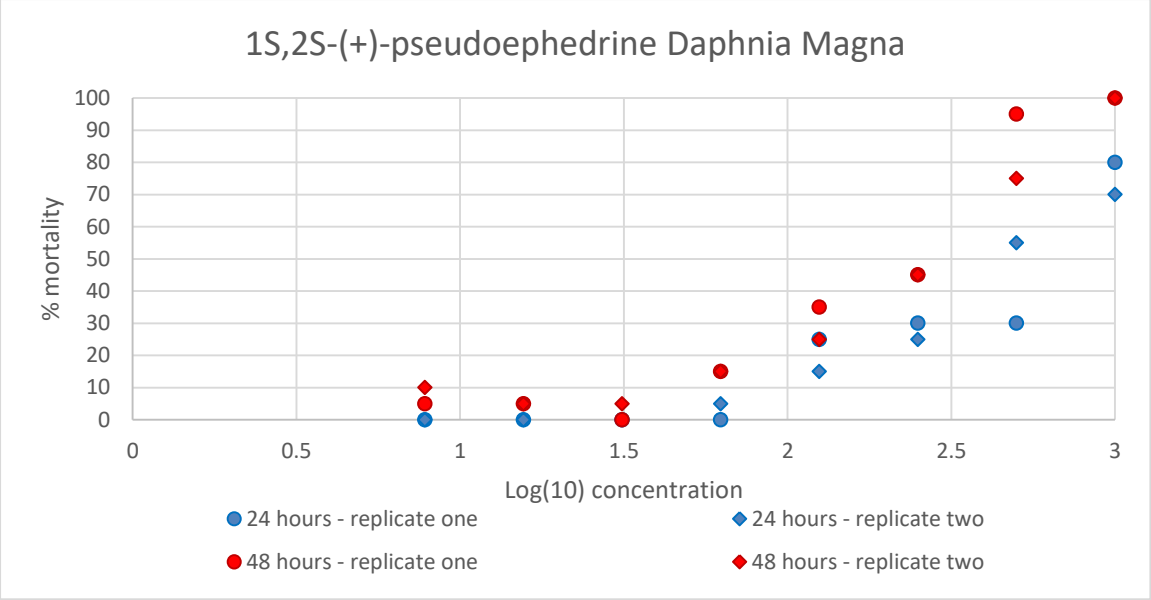


Figure S10. % mortality of *Daphnia Magna* exposed to 1S,2S-(+)-pseudoephedrine

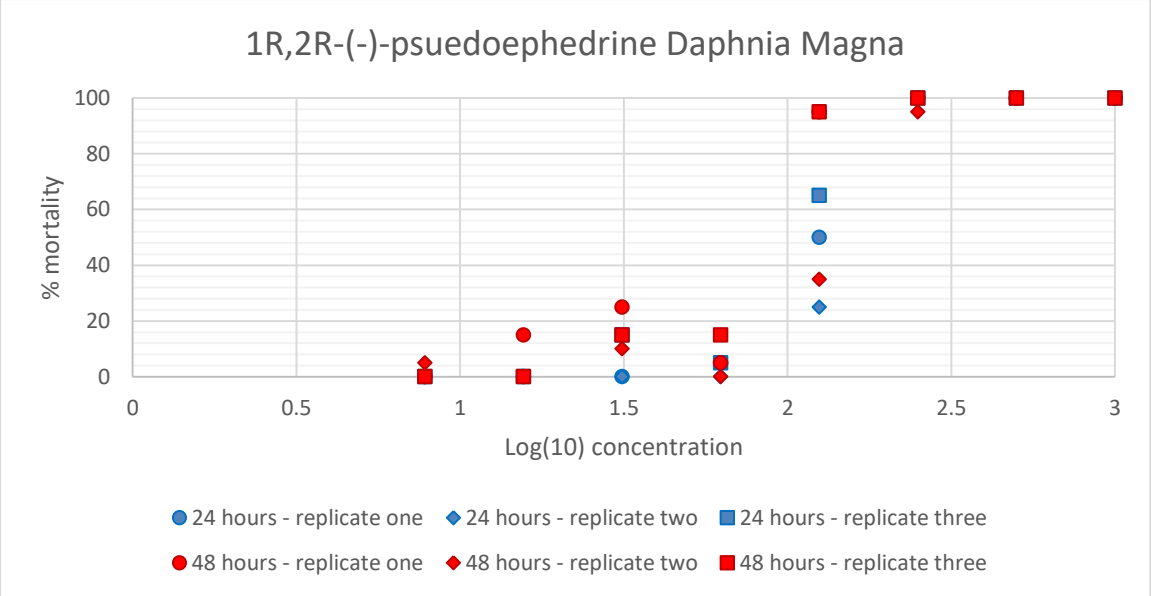


Figure S11. % mortality of *Daphnia Magna* exposed to 1R,2R-(-)-pseudoephedrine

Table S6. % mortality of *Daphnia Magna* at various concentrations after 24 hours

24 hour % mortality	Replicate	1000 mg/L	500 mg/L	250 mg/L	125 mg/L	62.5 mg/L	31.25 mg/L	15.625 mg/L	7.8125 mg/L
1S,2R -(+)- ephedrine	A	90	70	25	30	0	5	0	0
	B	95	60	35	40	0	5	0	0
1R,2S -(-)- ephedrine	A	100	93	43	18	7	4	7	7
	B	100	100	55	25	15	5	0	0
1S,2S -(+)- pseudo ephedrine	A	80	30	30	25	0	0	0	0
	B	70	55	25	15	5	0	0	0
1R,2R -(-)-pseudo ephedrine	A	100	100	100	50	5	0	0	0
	B	100	100	100	25	0	0	0	0
	C	100	100	100	65	5	15	0	0

Table S7. % mortality of *Daphnia Magna* at various concentrations after 48 hours

48 hour % mortality	Replicate	1000 mg/L	500 mg/L	250 mg/L	125 mg/L	62.5 mg/L	31.25 mg/L	15.625 mg/L	7.8125 mg/L
1S,2R -(+)- ephedrine	A	100	100	60	40	20	5	0	0
	B	100	100	65	40	40	5	0	0
1R,2S -(-)- ephedrine	A	100	93	43	18	7	4	7	7
	B	100	100	55	25	15	5	0	0
1S,2S -(+)- pseudo ephedrine	A	100	95	45	35	15	0	5	5
	B	100	75	45	25	15	5	5	10
1R,2R -(-)-pseudo ephedrine	A	100	100	100	95	5	25	15	0
	B	100	100	95	35	0	10	0	5
	C	100	100	100	95	15	15	0	0

Table S8. Ephedrine EC50 determination for *Daphnia Magna* after 24 hours exposure

Compound	Replicate	M	C	EC 50 (mg/L)	Average (mg/L)
1S,2R-(+)-ephedrine	A	149.5	-33.5	367.4	373.1
	B	83.0	-164.1	378.9	
1R,2S-(-)-ephedrine	A	142.4	-323.5	420.4	408.6
	B	99.7	-209.0	396.8	
1S,2S-(+)-pseudo ephedrine	A	166.1	-418.3	659.7	528.3
	B	49.8	-79.5	396.9	
1R,2R-(-)-pseudo ephedrine	A	157.8	-279.2	122.0	128.2
	B	249.1	-497.4	157.5	
	C	199.3	-353.0	105.1	

Table S9. Ephedrine EC50 determination for *Daphnia Magna* after 48 hours exposure

Compound	Replicate	M	C	EC 50 (mg/L)	Average (mg/L)
1S,2R-(+)-ephedrine	A	66.4	-99.3	176.8	170.8
	B	83.0	-134.14	164.9	
1R,2S-(-)-ephedrine	A	166.1	-355.4	276.0	253.7
	B	149.5	-303.5	231.4	
1S,2S-(+)-pseudo ephedrine	A	166.1	-353.3	267.9	274.3
	B	99.7	-194.0	280.6	
1R,2R-(-)-pseudo ephedrine	A	299.0	-531.9	88.4	107.2
	B	199.3	-383.0	148.6	
	C	265.8	-462.3	84.6	

Table S10. Multi-compound microcosm experiment –Biotic Light microcosms

Microcosm	Day	(+)-Eph (µg/L)	(-)-Eph (µg/L)	(+)-Pse (µg/L)	(-)-Pse (µg/L)	(+)-NorEph (ug/L)	(-)-NorEph (ug.L)	pH	Temp(°C)	DO (mg/l)
Biotic Light 1	0	0.74	0.84	0.45	0.48	0.86	0.47	N/A	N/A	N/A
Biotic Light 1	1	0.83	0.82	0.54	0.51	1.02	0.48	8.78	27.7	6.75
Biotic Light 1	4	0.68	0.66	0.43	0.44	0.99	0.38	8.62	28.6	6.46
Biotic Light 1	5	0.69	0.63	0.42	0.47	0.82	0.31	8.84	29	6.33
Biotic Light 1	6	0.62	0.00	0.00	0.33	0.47	0.23	8.94	30.2	7.29
Biotic Light 1	7	0.65	0.46	0.29	0.36	0.58	0.20	8.69	29.4	7.55
Biotic Light 1	8	0.71	0.46	0.23	0.39	0.74	0.22	8.51	29.3	7.48
Biotic Light 1	11	0.69	0.27	0.13	0.44	0.61	0.20	8.39	28.1	7.62
Biotic Light 1	12	0.70	0.21	0.07	0.31	0.45	0.18	8.36	29	6.72
Biotic Light 1	13	0.61	0.16	0.07	0.36	0.42	0.17	8.38	29.3	6.73
Biotic Light 1	14	0.61	0.15	0.04	0.32	0.41	0.16	8.15	29.2	6.74
Biotic Light 2	0	0.76	0.83	0.39	0.41	0.59	0.37	N/A	N/A	N/A
Biotic Light 2	1	0.75	0.74	0.36	0.36	0.60	0.27	8.8	27.3	6.86
Biotic Light 2	4	0.69	0.73	0.35	0.35	0.53	0.19	8.58	28.1	6.63
Biotic Light 2	5	0.68	0.64	0.36	0.37	0.55	0.19	8.62	28.6	6.45
Biotic Light 2	6	0.74	0.69	0.37	0.45	0.61	0.18	8.55	29.5	7.53
Biotic Light 2	7	0.70	0.63	0.34	0.37	0.61	0.19	8.46	29	7.71
Biotic Light 2	8	0.70	0.56	0.29	0.37	0.61	0.20	8.43	28.7	7.59
Biotic Light 2	11	0.71	0.44	0.25	0.37	0.56	0.20	8.4	27.7	7.77
Biotic Light 2	12	0.74	0.41	0.18	0.33	0.57	0.19	8.37	28.6	6.96
Biotic Light 2	13	0.69	0.40	0.21	0.38	0.51	0.19	8.43	29	6.81
Biotic Light 2	14	0.70	0.38	0.19	0.35	0.42	0.18	8.15	28.8	6.85

Table S11. Multi-compound microcosm experiment – Biotic Dark microcosms

Microcosm	Day	(+)-Eph (µg/L)	(-)-Eph (µg/L)	(+)-Pse (µg/L)	(-)-Pse (µg/L)	(+)-NorEph (µg/L)	(-)-NorEph (µg/L)	pH	Temp (°C)	DO (mg/l)
Biotic Dark 1	0	1.23	1.40	0.90	0.87	1.42	0.68	N/A	N/A	N/A
Biotic Dark 1	1	0.73	0.78	0.42	0.42	0.80	0.37	8.81	27.7	6.72
Biotic Dark 1	4	0.70	0.62	0.36	0.45	0.87	0.27	8.44	29	6.35
Biotic Dark 1	5	0.76	0.55	0.33	0.51	0.91	0.26	8.51	29.3	6.26
Biotic Dark 1	6	0.70	0.23	0.09	0.42	0.72	0.18	8.47	31	7.07
Biotic Dark 1	7	0.83	0.09	0.12	0.45	0.92	0.18	8.51	30.4	7.3
Biotic Dark 1	8	0.80	0.00	0.00	0.47	0.90	0.18	8.52	29.7	7.53
Biotic Dark 1	11	0.66	0.00	0.00	0.46	0.64	0.17	8.52	29.2	7.26
Biotic Dark 1	12	0.73	0.00	0.02	0.35	0.43	0.15	8.5	29.5	6.89
Biotic Dark 1	13	0.52	0.00	0.00	0.31	0.40	0.14	8.48	30.5	6.67
Biotic Dark 1	14	0.60	0.00	0.00	0.32	0.51	0.15	8.26	30.2	6.5
Biotic Dark 2	0	0.62	0.72	0.32	0.34	0.57	0.36	N/A	N/A	N/A
Biotic Dark 2	1	0.66	0.73	0.30	0.29	0.54	0.28	8.81	27.3	6.84
Biotic Dark 2	4	0.68	0.58	0.18	0.28	0.58	0.21	8.45	28.6	6.46
Biotic Dark 2	5	0.86	0.56	0.20	0.34	0.67	0.19	9.43	29.1	6.3
Biotic Dark 2	6	0.62	0.17	0.05	0.31	0.43	0.15	8.45	30.2	7.31
Biotic Dark 2	7	0.70	0.00	0.00	0.36	0.62	0.15	8.5	29.5	7.53
Biotic Dark 2	8	0.65	0.00	0.00	0.36	0.54	0.15	8.52	29.3	7.2
Biotic Dark 2	11	0.58	0.00	0.00	0.30	0.41	0.15	8.51	28.4	7.53
Biotic Dark 2	12	0.62	0.00	0.00	0.33	0.46	0.15	8.51	29.1	6.86
Biotic Dark 2	13	0.56	0.00	0.00	0.32	0.37	0.15	8.49	29.4	6.94
Biotic Dark 2	14	0.60	0.00	0.22	0.35	0.37	0.14	8.25	29.2	7.11

Table S12. Multi-compound microcosm experiment – Abiotic light microcosms

Microcosm	Day	(+)-Eph (µg/L)	(-)-Eph (µg/L)	(+)-Pse (µg/L)	(-)-Pse (µg/L)	(+)-NorEph (µg/L)	(-)-NorEph (µg/L)	pH	Temp (°C)	DO (mg/l)
Abiotic Light 1	0	0.64	0.75	0.30	0.35	0.51	0.34	N/A	N/A	N/A
Abiotic Light 1	1	0.70	0.78	0.34	0.36	0.54	0.37	8.86	27.1	6.92
Abiotic Light 1	4	0.69	0.79	0.29	0.33	0.66	0.40	8.56	28.3	6.56
Abiotic Light 1	5	0.62	0.76	0.41	0.41	0.70	0.42	8.54	28.8	6.41
Abiotic Light 1	6	0.58	0.70	0.29	0.35	0.60	0.38	8.51	30	7.35
Abiotic Light 1	7	0.74	0.84	0.29	0.35	0.79	0.44	8.54	29.3	7.6
Abiotic Light 1	8	0.66	0.70	0.28	0.28	0.66	0.38	8.55	28.9	7.45
Abiotic Light 1	11	0.56	0.66	0.26	0.28	0.57	0.36	8.55	28.1	7.62
Abiotic Light 1	12	0.77	0.75	0.42	0.40	0.84	0.44	8.53	28.4	6.97
Abiotic Light 1	13	0.69	0.73	0.32	0.35	0.80	0.45	8.53	29.3	6.72
Abiotic Light 1	14	0.64	0.68	0.35	0.37	0.74	0.43	8.3	28.9	6.82
Abiotic Light 2	0	0.72	0.86	0.43	0.34	0.80	0.46	N/A	N/A	N/A
Abiotic Light 2	1	0.73	0.74	0.39	0.38	0.64	0.39	8.84	27	6.93
Abiotic Light 2	5	0.88	0.99	0.34	0.38	0.93	0.49	8.47	28.9	6.38
Abiotic Light 2	6	0.69	0.79	0.38	0.36	0.69	0.42	8.46	30	7.34
Abiotic Light 2	7	0.69	0.73	0.33	0.32	0.81	0.46	8.51	29.3	7.57
Abiotic Light 2	8	0.62	0.67	0.37	0.36	0.57	0.39	8.53	28.9	7.34
Abiotic Light 2	11	0.67	0.78	0.36	0.40	1.04	0.52	8.54	28.2	7.61
Abiotic Light 2	12	0.77	0.73	0.47	0.45	0.93	0.52	8.52	28.8	6.97
Abiotic Light 2	13	0.73	0.72	0.48	0.44	1.06	0.56	8.52	29.4	6.68
Abiotic Light 2	14	0.79	0.83	0.50	0.52	1.23	0.59	8.29	29.1	6.75

Table S13. Multi-compound microcosm experiment – Abiotic Dark microcosms

Microcosm	Day	(+)-Eph (µg/L)	(-)-Eph (µg/L)	(+)-Pse (µg/L)	(-)-Pse (µg/L)	(+)-NorEph (µg/L)	(-)-NorEph (µg/L)	pH	Temp (°C)	DO (mg/l)
Abiotic Dark 1	0	0.79	1.15	0.39	0.40	0.81	0.47	N/A	N/A	N/A
Abiotic Dark 1	1	0.60	0.71	0.30	0.34	0.50	0.36	8.86	27.5	6.83
Abiotic Dark 1	4	0.67	0.68	0.34	0.31	0.62	0.38	8.46	29.3	6.29
Abiotic Dark 1	5	0.74	0.80	0.37	0.34	0.65	0.41	8.51	27.8	6.7
Abiotic Dark 1	6	0.86	0.91	0.36	0.35	0.83	0.50	8.54	29.1	7.48
Abiotic Dark 1	7	0.90	0.89	0.37	0.36	0.71	0.44	8.55	28.8	7.76
Abiotic Dark 1	8	0.75	0.57	0.32	0.31	0.65	0.40	8.55	27.9	7.62
Abiotic Dark 1	11	0.75	0.70	0.43	0.43	0.78	0.43	8.51	27.6	7.37
Abiotic Dark 1	12	0.69	0.57	0.37	0.40	0.77	0.39	8.52	27.6	7.35
Abiotic Dark 1	13	0.67	0.37	0.34	0.36	0.81	0.32	8.51	28.9	6.84
Abiotic Dark 1	14	0.66	0.00	0.46	0.44	0.63	0.17	8.68	25.2	7.77
Abiotic Dark 2	0	0.63	0.76	0.53	0.49	0.53	0.35	N/A	N/A	N/A
Abiotic Dark 2	1	0.91	1.07	0.48	0.55	0.89	0.48	8.85	27.6	6.78
Abiotic Dark 2	4	0.57	0.62	0.31	0.34	0.55	0.35	8.48	27.9	6.7
Abiotic Dark 2	5	0.67	0.69	0.36	0.36	0.56	0.38	8.51	27.5	6.81
Abiotic Dark 2	6	0.60	0.69	0.29	0.32	0.58	0.38	8.53	29.2	7.41
Abiotic Dark 2	7	0.65	0.70	0.34	0.34	0.52	0.36	8.56	28.6	7.93
Abiotic Dark 2	8	0.66	0.69	0.30	0.36	0.54	0.36	8.56	27.6	7.69
Abiotic Dark 2	11	0.68	0.70	0.29	0.35	0.64	0.39	8.56	27	7.62
Abiotic Dark 2	12	0.66	0.70	0.32	0.35	0.61	0.38	8.55	27.2	7.36
Abiotic Dark 2	13	0.74	0.76	0.30	0.38	0.65	0.40	8.52	28.5	7.32
Abiotic Dark 2	14	0.71	0.80	0.36	0.38	0.58	0.40	8.85	26.4	7.22

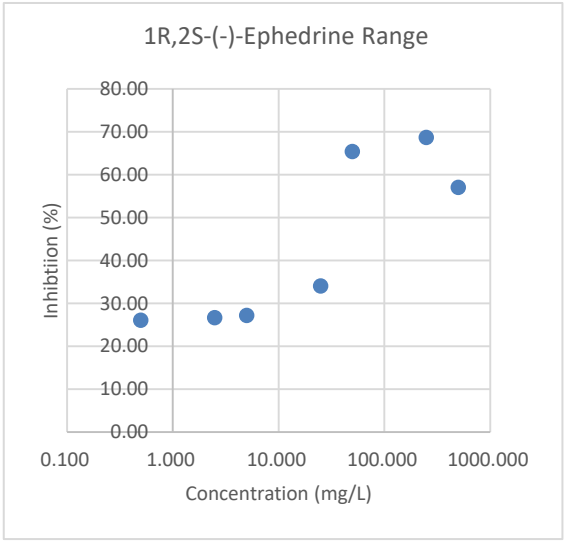
Table S14. 1R,2S-(-)-Ephedrine range finding test - *Tetrahymena thermophile*

Results – Optical Density

Conc.	Time	Replicate		Mean	Std. dev.	CV%
		1	2			
	t0	0.711	0.811	0.761	0.071	9.29%
Control	t24	0.192	0.281	0.237	0.063	26.61%
	t0	0.777	0.755	0.766	0.016	2.03%
0.500	t24	0.409	0.347	0.378	0.044	11.60%
	t0	0.777	0.765	0.771	0.008	1.10%
2.500	t24	0.419	0.353	0.386	0.047	12.09%
	t0	0.770	0.775	0.773	0.004	0.46%
5.000	t24	0.405	0.376	0.391	0.021	5.25%
	t0	0.778	0.766	0.772	0.008	1.10%
25.000	t24	0.424	0.428	0.426	0.003	0.66%
	t0	0.765	0.767	0.766	0.001	0.18%
50.000	t24	0.579	0.590	0.585	0.008	1.33%
	t0	0.770	0.766	0.768	0.003	0.37%
250.000	t24	0.597	0.610	0.604	0.009	1.52%
	t0	0.775	0.766	0.771	0.006	0.83%
500.000	t24	0.540	0.550	0.545	0.007	1.30%

Summary of Results

Conc.	Statistics	Time (hours)	
		0	24
Control	Mean	0.761	0.237
	CV%	9.29%	26.61%
0.500	Mean	0.766	0.378
	CV%	2.03%	11.60%
2.500	Mean	0.771	0.386
	CV%	1.10%	12.09%
5.000	Mean	0.773	0.391
	CV%	0.46%	5.25%
25.000	Mean	0.772	0.426
	CV%	1.10%	0.66%
50.000	Mean	0.766	0.585
	CV%	0.18%	1.33%
250.000	Mean	0.768	0.604
	CV%	0.37%	1.52%
500.000	Mean	0.771	0.545
	CV%	0.83%	1.30%



Percentage Inhibition Computation

Conc.	0	100%	% I
Control	0.237	0.525	0.000
0.500			26.025
2.500			26.597
5.000			27.169
25.000			34.032
50.000			65.396
250.000			68.637

500.000 57.007

Concentration vs. Percent Inhibition

Log Conc.	I%	Conc.
-0.301	26.02	0.500
0.398	26.60	2.500
0.699	27.17	5.000
1.398	34.03	25.000
1.699	65.40	50.000
2.398	68.64	250.000
2.699	57.01	500.000

Effect Concentration Results

log x =	**-0.925**
24hEC10 =	**0.119**
log x =	**-0.266**
24hEC20 =	**0.542**
log x =	**1.709**
24hEC50 =	**50.919**
log x =	**3.026**
24hEC70 =	**1052.423**
log x =	**4.342**
24hEC90 =	**21752.161**

Lower 95%	Upper 95%
6.622	41.470

Summary Output

Regression Statistics	
Multiple R	0.853
R Square	0.728
Adjusted R Square	0.673
Standard Error	11.051
Observations	7

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	1630.479	1630.479	13.350	0.015
Residual	5	610.658	122.132		
Total	6	2241.136			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	24.046	6.778	3.547	0.016	6.622	41.470	6.622	41.470
X Variable 1	15.188	4.157	3.654	0.015	4.503	25.874	4.503	25.874

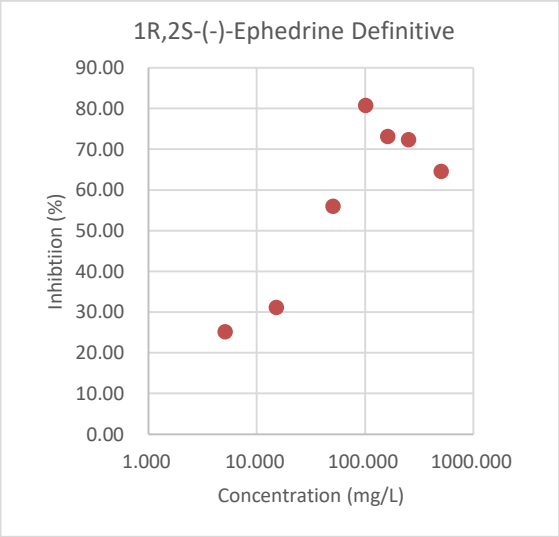
Table S15. 1R,2S-(-)-Ephedrine definitive test - *Tetrahymena thermophila*

Results – Optical Density

Conc.	Time	Replicate		Mean	Std. dev.	CV%
		1	2			
	t0	0.751	0.720	0.736	0.022	2.98%
0.000	t24	0.200	0.237	0.219	0.026	11.97%
	t0	0.754	0.753	0.754	0.001	0.09%
5.100	t24	0.324	0.409	0.367	0.060	16.40%
	t0	0.744	0.745	0.745	0.001	0.09%
15.200	t24	0.375	0.402	0.389	0.019	4.91%
	t0	0.738	0.744	0.741	0.004	0.57%
50.600	t24	0.460	0.567	0.514	0.076	14.73%
	t0	0.749	0.755	0.752	0.004	0.56%
101.100	t24	0.646	0.659	0.653	0.009	1.41%
	t0	0.741	0.735	0.738	0.004	0.57%
161.800	t24	0.599	0.599	0.599	0.000	0.00%
	t0	0.740	0.740	0.740	0.000	0.00%
252.800	t24	0.600	0.594	0.597	0.004	0.71%
	t0	0.738	0.734	0.736	0.003	0.38%
505.600	t24	0.550	0.556	0.553	0.004	0.77%

Summary of Results

Conc.	Statistics	Time (hours)	
		0	24
0.00	Mean	0.736	0.219
	CV%	2.98%	11.97%
5.100	Mean	0.754	0.367
	CV%	0.09%	16.40%
15.200	Mean	0.745	0.389
	CV%	0.09%	4.91%
50.600	Mean	0.741	0.514
	CV%	0.57%	14.73%
101.100	Mean	0.752	0.653
	CV%	0.56%	1.41%
161.800	Mean	0.738	0.599
	CV%	0.57%	0.00%
252.800	Mean	0.740	0.597
	CV%	0.00%	0.71%
505.600	Mean	0.736	0.553
	CV%	0.38%	0.77%



Percentage Inhibition Computation

Conc.	0	100%	% I
0.00	0.219	0.517	0.000
5.100			25.145
15.200			31.141
50.600			55.996
101.100			80.754
161.800			73.114
252.800			72.340
505.600			64.603

Concentration vs. Percent Inhibition

Log Conc.	I%	Conc.
0.708	25.15	5.100
1.182	31.14	15.200
1.704	56.00	50.600
2.005	80.75	101.100
2.209	73.11	161.800
2.403	72.34	252.800
2.704	64.60	505.600

Effect Concentration Results

log x =	**0.045**
24hEC10 =	**1.110**
log x =	**0.423**
24hEC20 =	**2.648**
log x =	**1.558**
24hEC50 =	**35.995**
log x =	**2.314**
24hEC70 =	**205.001**
log x =	**3.071**
24hEC90 =	**1167.546**

Lower 95%	Upper 95%
-26.498	44.105

Summary Output

Regression Statistics	
Multiple R	0.860
R Square	0.739
Adjusted R Square	0.687
Standard Error	12.079
Observations	7

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	2069.623	2069.623	14.186	0.013
Residual	5	729.479	145.896		
Total	6	2799.102			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	8.803	13.733	0.641	0.550	-26.498	44.105	-26.498	44.105
X Variable 1	26.442	7.021	3.766	0.013	8.395	44.489	8.395	44.489

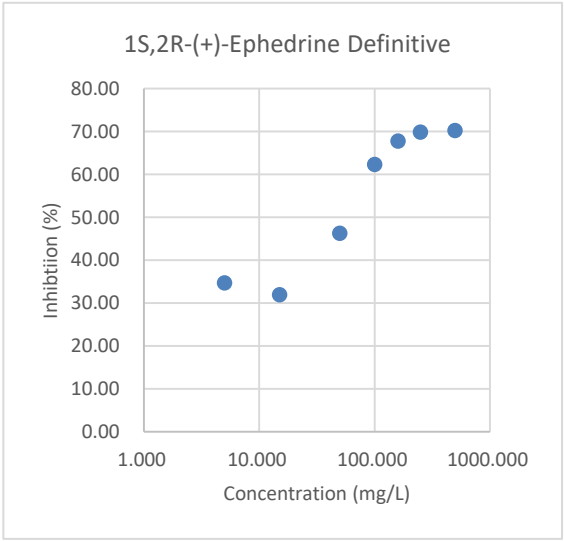
Table S16. 1S,2R-(+)-Ephedrine definitive test - *Tetrahymena thermophila*

Results – Optical Density

Conc.	Time	Replicate		Mean	Std. dev.	CV%
		1	2			
	t0	0.777	0.756	0.767	0.015	1.94%
Control	t24	0.245	0.290	0.268	0.032	11.90%
	t0	0.771	0.781	0.776	0.007	0.91%
5.024	t24	0.446	0.454	0.450	0.006	1.26%
	t0	0.760	0.763	0.762	0.002	0.28%
15.072	t24	0.429	0.415	0.422	0.010	2.35%
	t0	0.764	0.756	0.760	0.006	0.74%
50.240	t24	0.484	0.500	0.492	0.011	2.30%
	t0	0.771	0.761	0.766	0.007	0.92%
100.480	t24	0.593	0.563	0.578	0.021	3.67%
	t0	0.778	0.773	0.776	0.004	0.46%
160.768	t24	0.615	0.614	0.615	0.001	0.12%
	t0	0.756	0.774	0.765	0.013	1.66%
251.200	t24	0.607	0.622	0.615	0.011	1.73%
	t0	0.759	0.752	0.756	0.005	0.66%
502.400	t24	0.614	0.600	0.607	0.010	1.63%

Summary of Results

Conc.	Statistics	Time (hours)	
		0	24
Control	Mean	0.767	0.268
	CV%	1.94%	11.90%
5.024	Mean	0.776	0.450
	CV%	0.91%	1.26%
15.072	Mean	0.762	0.422
	CV%	0.28%	2.35%
50.240	Mean	0.760	0.492
	CV%	0.74%	2.30%
100.480	Mean	0.766	0.578
	CV%	0.92%	3.67%
160.768	Mean	0.776	0.615
	CV%	0.46%	0.12%
251.200	Mean	0.765	0.615
	CV%	1.66%	1.73%
502.400	Mean	0.756	0.607
	CV%	0.66%	1.63%



Percentage Inhibition Computation

Conc.	0	100%	% I
Control	0.268	0.499	0.000
5.024			34.669
15.072			31.964
50.240			46.293
100.480			62.325
160.768			67.735
251.200			69.840
502.400			70.240

Concentration vs. Percent Inhibition

Log Conc.	I%	Conc.
0.701	34.67	5.024
1.178	31.96	15.072
1.701	46.29	50.240
2.002	62.32	100.480
2.206	67.74	160.768
2.400	69.84	251.200
2.701	70.24	502.400

Effect Concentration Results

log x =	**-0.144**
24hEC10 =	**0.718**
log x =	**0.300**
24hEC20 =	**1.993**
log x =	**1.632**
24hEC50 =	**42.642**
log x =	**2.520**
24hEC70 =	**328.646**
log x =	**3.407**
24hEC90 =	**2532.922**

Lower 95%	Upper 95%
-4.269	30.761

Summary Output

Regression Statistics	
Multiple R	0.945
R Square	0.893
Adjusted R Square	0.872
Standard Error	6.012
Observations	7

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	1506.905	1506.905	41.695	0.001
Residual	5	180.707	36.141		
Total	6	1687.612			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	13.246	6.814	1.944	0.109	-4.269	30.761	-4.269	30.761
X Variable 1	22.525	3.488	6.457	0.001	13.558	31.493	13.558	31.493

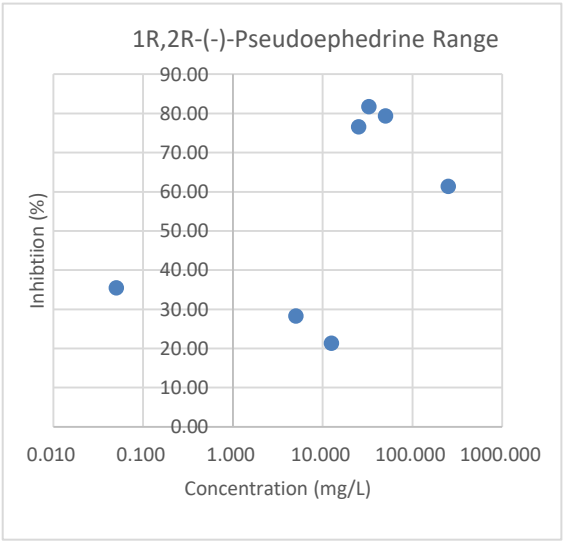
Table S17. 1R,2R-(-)-Pseudoephedrine range finding test - *Tetrahymena thermophila*

Results – Optical Density

Conc.	Time	Replicate		Mean	Std. dev.	CV%
		1	2			
	t0	0.757	0.746	0.752	0.008	1.04%
0.000	t24	0.272	0.251	0.262	0.015	5.68%
	t0	0.751	0.748	0.750	0.002	0.28%
0.005	t24	0.297	0.311	0.304	0.010	3.26%
	t0	0.737	0.742	0.740	0.004	0.48%
0.050	t24	0.342	0.357	0.350	0.011	3.03%
	t0	0.734	0.745	0.740	0.008	1.05%
0.502	t24	0.300	0.348	0.324	0.034	10.48%
	t0	0.758	0.748	0.753	0.007	0.94%
5.024	t24	0.364	0.302	0.333	0.044	13.17%
	t0	0.741	0.686	0.714	0.039	5.45%
50.240	t24	0.554	0.538	0.546	0.011	2.07%
	t0	0.733	0.742	0.738	0.006	0.86%
251.200	t24	0.606	0.612	0.609	0.004	0.70%
	t0	0.737	0.720	0.729	0.012	1.65%
502.400	t24	0.529	0.569	0.549	0.028	5.15%

Summary of Results

Conc.	Statistics	Time (hours)	
		0	24
0.00	Mean	0.752	0.262
	CV%	1.04%	5.68%
0.005	Mean	0.750	0.304
	CV%	0.28%	3.26%
0.050	Mean	0.740	0.350
	CV%	0.48%	3.03%
0.502	Mean	0.740	0.324
	CV%	1.05%	10.48%
5.024	Mean	0.753	0.333
	CV%	0.94%	13.17%
50.240	Mean	0.714	0.546
	CV%	5.45%	2.07%
251.200	Mean	0.738	0.609
	CV%	0.86%	0.70%
502.400	Mean	0.729	0.549
	CV%	1.65%	5.15%



Percentage Inhibition Computation

Conc.	0	100%	% I
0.00	0.262	0.490	0.000
0.005			9.082
0.050			20.408
0.502			15.204
5.024			14.286
50.240			65.816
251.200			73.776
502.400			63.367

Concentration vs. Percent Inhibition

Log Conc.	I%	Conc.
-2.299	9.08	0.005
-1.299	20.41	0.050
-0.299	15.20	0.502
0.701	14.29	5.024
1.701	65.82	50.240
2.400	73.78	251.200
2.701	63.37	502.400

Effect Concentration Results

log x =	**-1.580**
24hEC10 =	**0.026**
log x =	**-0.816**
24hEC20 =	**0.153**
log x =	**1.476**
24hEC50 =	**29.829**
log x =	**3.004**
24hEC70 =	**1002.068**
log x =	**4.532**
24hEC90 =	**33663.146**

Lower 95%	Upper 95%
14.899	46.453

Summary Output

Regression Statistics	
Multiple R	0.868
R Square	0.753
Adjusted R Square	0.704
Standard Error	15.583
Observations	7

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	3708.343	3708.343	15.271	0.011
Residual	5	1214.204	242.841		
Total	6	4922.547			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	30.676	6.138	4.998	0.004	14.899	46.453	14.899	46.453
X Variable 1	13.089	3.350	3.908	0.011	4.479	21.700	4.479	21.700

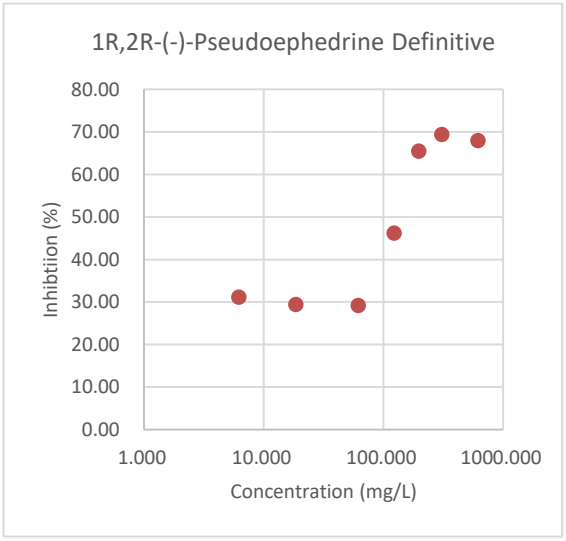
Table S18. 1R,2R-(-)-Pseudoephedrine definitive test - *Tetrahymena thermophila*

Results – Optical Density

Conc.	Time	Replicate		Mean	Std. dev.	CV%
		1	2			
	t0	0.773	0.787	0.780	0.010	1.27%
0.000	t24	0.283	0.256	0.270	0.019	7.08%
	t0	0.772	0.775	0.774	0.002	0.27%
0.050	t24	0.392	0.496	0.444	0.074	16.56%
	t0	0.783	0.766	0.775	0.012	1.55%
5.020	t24	0.350	0.467	0.409	0.083	20.25%
	t0	0.764	0.778	0.771	0.010	1.28%
12.550	t24	0.375	0.364	0.370	0.008	2.11%
	t0	0.774	0.767	0.771	0.005	0.64%
25.100	t24	0.662	0.640	0.651	0.016	2.39%
	t0	0.751	0.774	0.763	0.016	2.13%
32.630	t24	0.660	0.679	0.670	0.013	2.01%
	t0	0.772	0.764	0.768	0.006	0.74%
50.200	t24	0.664	0.661	0.663	0.002	0.32%
	t0	0.767	0.763	0.765	0.003	0.37%
251.000	t24	0.557	0.579	0.568	0.016	2.74%

Summary of Results

Conc.	Statistics	Time (hours)	
		0	24
0.00	Mean	0.780	0.270
	CV%	1.27%	7.08%
0.050	Mean	0.774	0.444
	CV%	0.27%	16.56%
5.020	Mean	0.775	0.409
	CV%	1.55%	20.25%
12.550	Mean	0.771	0.370
	CV%	1.28%	2.11%
25.100	Mean	0.771	0.651
	CV%	0.64%	2.39%
32.630	Mean	0.763	0.670
	CV%	2.13%	2.01%
50.200	Mean	0.768	0.663
	CV%	0.74%	0.32%
251.000	Mean	0.765	0.568
	CV%	0.37%	2.74%



Percentage Inhibition Computation

Conc.	0	100%	% I
0.00	0.270	0.511	0.000
0.050			35.455
5.020			28.306
12.550			21.352
25.100			76.592
32.630			81.783
50.200			79.334
251.000			61.410

Concentration vs. Percent Inhibition

Log Conc.	I%	Conc.
-1.299	35.46	0.050
0.701	28.31	5.020
1.099	21.35	12.550
1.400	76.59	25.100
1.514	81.78	32.630
1.701	79.33	50.200
2.400	61.41	251.000

Effect Concentration Results

log x =	**-2.690**
24hEC10 =	**0.002**
log x =	**-1.852**
24hEC20 =	**0.014**
log x =	**0.663**
24hEC50 =	**4.599**
log x =	**2.340**
24hEC70 =	**217.511**
log x =	**4.017**
24hEC90 =	**10286.829**

Lower 95%	Upper 95%
9.304	74.869

Summary Output

Regression Statistics	
Multiple R	0.538
R Square	0.289
Adjusted R Square	0.147
Standard Error	23.973
Observations	7

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	1169.863	1169.863	2.036	0.213
Residual	5	2873.586	574.717		
Total	6	4043.449			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	42.086	12.753	3.300	0.021	9.304	74.869	9.304	74.869
X Variable 1	11.928	8.361	1.427	0.213	-9.563	33.420	-9.563	33.420

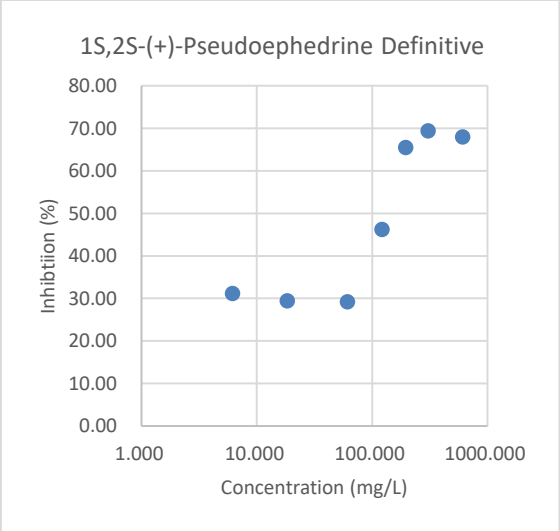
Table S19. 1S,2S-(+)-Pseudoephedrine definitive test - *Tetrahymena thermophila*

Results – Optical Density

Conc.	Time	Replicate		Mean	Std. dev.	CV%
		1	2			
	t0	0.750	0.760	0.755	0.007	0.94%
0.000	t24	0.233	0.253	0.243	0.014	5.82%
	t0	0.750	0.753	0.752	0.002	0.28%
6.200	t24	0.401	0.397	0.399	0.003	0.71%
	t0	0.763	0.762	0.763	0.001	0.09%
18.500	t24	0.402	0.400	0.401	0.001	0.35%
	t0	0.755	0.766	0.761	0.008	1.02%
61.500	t24	0.363	0.433	0.398	0.049	12.44%
	t0	0.753	0.753	0.753	0.000	0.00%
123.000	t24	0.405	0.550	0.478	0.103	21.47%
	t0	0.761	0.764	0.763	0.002	0.28%
196.800	t24	0.593	0.578	0.586	0.011	1.81%
	t0	0.748	0.752	0.750	0.003	0.38%
307.500	t24	0.592	0.594	0.593	0.001	0.24%
	t0	0.753	0.753	0.753	0.000	0.00%
615.000	t24	0.563	0.615	0.589	0.037	6.24%

Summary of Results

Conc.	Statistics	Time (hours)	
		0	24
0.00	Mean	0.755	0.243
	CV%	0.94%	5.82%
6.200	Mean	0.752	0.399
	CV%	0.28%	0.71%
18.500	Mean	0.763	0.401
	CV%	0.09%	0.35%
61.500	Mean	0.761	0.398
	CV%	1.02%	12.44%
123.000	Mean	0.753	0.478
	CV%	0.00%	21.47%
196.800	Mean	0.763	0.586
	CV%	0.28%	1.81%
307.500	Mean	0.750	0.593
	CV%	0.38%	0.24%
615.000	Mean	0.753	0.589
	CV%	0.00%	6.24%



Percentage Inhibition Computation

Conc.	0	100%	% I
0.00	0.243	0.512	0.000
6.200			31.152
18.500			29.395
61.500			29.199
123.000			46.191
196.800			65.430
307.500			69.336
615.000			67.969

Concentration vs. Percent Inhibition

Log Conc.	I%	Conc.
0.792	31.15	6.200
1.267	29.39	18.500
1.789	29.20	61.500
2.090	46.19	123.000
2.294	65.43	196.800
2.488	69.34	307.500
2.789	67.97	615.000

Effect Concentration Results

log x =	**0.289**
24hEC10 =	**1.945**
log x =	**0.717**
24hEC20 =	**5.200**
log x =	**1.999**
24hEC50 =	**99.265**
log x =	**2.854**
24hEC70 =	**709.061**
log x =	**3.709**
24hEC90 =	**5064.911**

Lower 95%	Upper 95%
-27.890	34.351

Summary Output

Regression Statistics	
Multiple R	0.869
R Square	0.756
Adjusted R Square	0.707
Standard Error	10.228
Observations	7

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	1620.413	1620.413	15.489	0.011
Residual	5	523.082	104.616		
Total	6	2143.495			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	3.231	12.106	0.267	0.800	-27.890	34.351	-27.890	34.351
X Variable 1	23.396	5.945	3.936	0.011	8.115	38.677	8.115	38.677

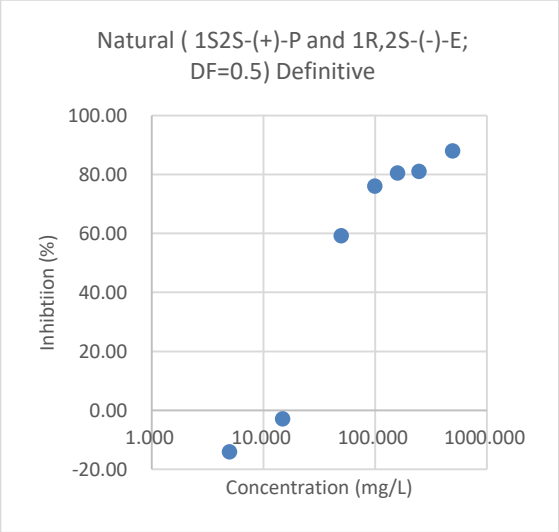
Table S20. 'Natural' isomers (1S,2S-(+)-Pseudoephedrine and 1R,2S-(-)-ephedrine) DF = 0.5 definitive test - *Tetrahymena thermophila*

Results – Optical Density

Conc.	Time	Replicate		Mean	Std. dev.	CV%
		1	2			
	t0	0.758	0.774	0.766	0.011	1.48%
0.000	t24	0.223	0.222	0.223	0.001	0.32%
	t0	0.750	0.765	0.758	0.011	1.40%
4.978	t24	0.156	0.119	0.138	0.026	19.03%
	t0	0.753	0.755	0.754	0.001	0.19%
14.933	t24	0.206	0.184	0.195	0.016	7.98%
	t0	0.767	0.753	0.760	0.010	1.30%
49.775	t24	0.592	0.485	0.539	0.076	14.05%
	t0	0.752	0.755	0.754	0.002	0.28%
99.550	t24	0.681	0.566	0.624	0.081	13.04%
	t0	0.744	0.746	0.745	0.001	0.19%
159.280	t24	0.645	0.633	0.639	0.008	1.33%
	t0	0.747	0.753	0.750	0.004	0.57%
248.875	t24	0.660	0.634	0.647	0.018	2.84%
	t0	0.751	0.757	0.754	0.004	0.56%
497.750	t24	0.592	0.785	0.689	0.136	19.82%

Summary of Results

Conc.	Statistics	Time (hours)	
		0	24
0.00	Mean	0.766	0.223
	CV%	1.48%	0.32%
4.978	Mean	0.758	0.138
	CV%	1.40%	19.03%
14.933	Mean	0.754	0.195
	CV%	0.19%	7.98%
49.775	Mean	0.760	0.539
	CV%	1.30%	14.05%
99.550	Mean	0.754	0.624
	CV%	0.28%	13.04%
159.280	Mean	0.745	0.639
	CV%	0.19%	1.33%
248.875	Mean	0.750	0.647
	CV%	0.57%	2.84%
497.750	Mean	0.754	0.689
	CV%	0.56%	19.82%



Percentage Inhibition Computation

Conc.	0	100%	% I
0.00	0.223	0.544	0.000
4.978			-14.075
14.933			-2.852
49.775			59.246
99.550			76.081
159.280			80.497
248.875			81.049

497.750	87.948

Concentration vs. Percent Inhibition

Log Conc.	I%	Conc.
0.697	-14.08	4.978
1.174	-2.85	14.933
1.697	59.25	49.775
1.998	76.08	99.550
2.202	80.50	159.280
2.396	81.05	248.875
2.697	87.95	497.750

Effect Concentration Results

log x =	**1.103**
24hEC10 =	**12.640**
log x =	**1.276**
24hEC20 =	**18.798**
log x =	**1.793**
24hEC50 =	**61.832**
log x =	**2.138**
24hEC70 =	**136.757**
log x =	**2.483**
24hEC90 =	**302.474**

Lower 95%	Upper 95%
-94.616	-13.219

Summary Output

Regression Statistics	
Multiple R	0.954
R Square	0.911
Adjusted R Square	0.893
Standard Error	13.996
Observations	7

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	9973.600	9973.600	50.911	0.001
Residual	5	979.509	195.902		
Total	6	10953.109			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	-53.917	15.832	-3.406	0.019	-94.616	-13.219	-94.616	13.219
X Variable 1	57.950	8.122	7.135	0.001	37.073	78.828	37.073	78.828

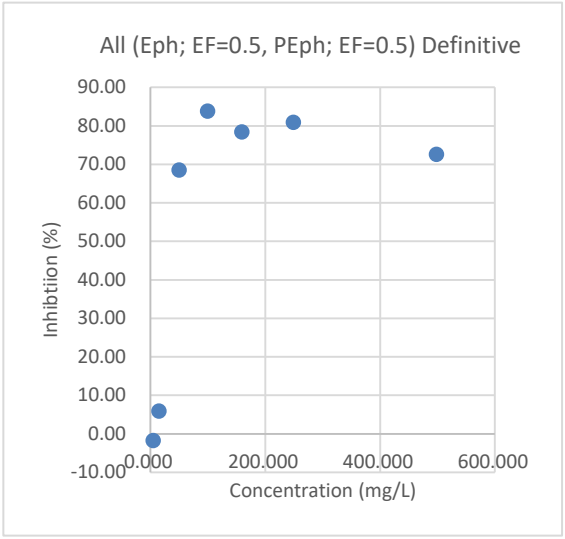
Table S21. 'All' isomers EF = 0.5 and DF = 0.5 definitive test - *Tetrahymena thermophila*

Results – Optical Density

Conc.	Time	Replicate		Mean	Std. dev.	CV%
		1	2			
	t0	0.759	0.761	0.760	0.001	0.19%
0.000	t24	0.165	0.162	0.164	0.002	1.30%
	t0	0.747	0.749	0.748	0.001	0.19%
4.981	t24	0.120	0.161	0.141	0.029	20.63%
	t0	0.760	0.754	0.757	0.004	0.56%
14.944	t24	0.225	0.166	0.196	0.042	21.34%
	t0	0.762	0.763	0.763	0.001	0.09%
49.813	t24	0.534	0.615	0.575	0.057	9.97%
	t0	0.746	0.758	0.752	0.008	1.13%
99.625	t24	0.644	0.666	0.655	0.016	2.38%
	t0	0.758	0.761	0.760	0.002	0.28%
159.400	t24	0.632	0.629	0.631	0.002	0.34%
	t0	0.760	0.755	0.758	0.004	0.47%
249.063	t24	0.664	0.622	0.643	0.030	4.62%
	t0	0.754	0.754	0.754	0.000	0.00%
498.125	t24	0.594	0.586	0.590	0.006	0.96%

Summary of Results

Conc.	Statistics	Time (hours)	
		0	24
0.00	Mean	0.760	0.164
	CV%	0.19%	1.30%
4.981	Mean	0.748	0.141
	CV%	0.19%	20.63%
14.944	Mean	0.757	0.196
	CV%	0.56%	21.34%
49.813	Mean	0.763	0.575
	CV%	0.09%	9.97%
99.625	Mean	0.752	0.655
	CV%	1.13%	2.38%
159.400	Mean	0.760	0.631
	CV%	0.28%	0.34%
249.063	Mean	0.758	0.643
	CV%	0.47%	4.62%
498.125	Mean	0.754	0.590
	CV%	0.00%	0.96%



Percentage Inhibition Computation

Conc.	0	100%	% I
0.00	0.164	0.597	0.000
4.981			-1.844
14.944			5.868
49.813			68.483
99.625			83.738
159.400			78.374
249.063			80.805
498.125			72.506

Concentration vs. Percent Inhibition

Log Conc.	I%	Conc.
0.697	-1.84	4.981
1.174	5.87	14.944
1.697	68.48	49.813
1.998	83.74	99.625
2.202	78.37	159.400
2.396	80.80	249.063
2.697	72.51	498.125

Effect Concentration Results

log x =	**0.861**
24hEC10 =	**7.246**
log x =	**1.076**
24hEC20 =	**11.882**
log x =	**1.721**
24hEC50 =	**52.387**
log x =	**2.151**
24hEC70 =	**140.856**
log x =	**2.581**
24hEC90 =	**378.727**

Lower 95%	Upper 95%
-84.400	24.306

Summary Output

Regression Statistics	
Multiple R	0.887
R Square	0.786
Adjusted R Square	0.743
Standard Error	18.690
Observations	7

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	6423.891	6423.891	18.391	0.008
Residual	5	1746.505	349.301		
Total	6	8170.396			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	-30.047	21.144	-1.421	0.215	-84.400	24.306	-84.400	24.306
X Variable 1	46.508	10.845	4.288	0.008	18.630	74.386	18.630	74.386

Additional data interpretation

Firstly, whilst a large portion of the introductory portion of the paper is rightly dedicated to a discussion of ephedrine's metabolism in mammals there was no discussion of its pharmacology. Ephedrine is a direct and indirect sympathomimetic amine [24], meaning it binds directly to alpha and beta adrenergic receptors (direct) whilst also inhibiting neuronal norepinephrine reuptake and displacing norepinephrine from storage vesicles (in-direct), which allows norepinephrine to bind to alpha and beta receptors [25]. Whilst this has no readily apparent bearing on ephedrine's metabolism, it is important to consider that its activity within humans may be related to its activity and subsequent toxicity to the species investigated in the paper: *D. magna, P. subcapitata* and *T. thermophila*.

The degradation of ephedrine isomers in the first river simulating microcosms was interpreted only by visual examination of the changes in EF and isomer concentration (as shown in figures 1 and 2). Whilst this was sufficient for the dark biotic reactors where (-)-1R, 2S-ephedrine and (+)-1S, 2S-pseudoephedrine were not detected at the end of the experiment, the changes in the other reactors would benefit from statistical analysis. To this end, a 2-tailed t-tail test was used to compare the average concentration in each pair of reactors, i.e. biotic dark 1 and biotic dark 2, on days 0-1 with the average concentration on days 11-14, using data presented in supplementary table S10-S13. For 1R,2S-(-)-ephedrine and 1S,2S-(+)-pseudoephedrine the concentration of both isomers decreased significantly (<0.01) in the biotic light and biotic dark microcosms, with no significant ($P>0.05$) change in concentration in the abiotic microcosms. For 1S,2R-(+)-ephedrine there was a significantly ($P<0.01$) decrease in concentration in the biotic light and ($P<0.05$) in the biotic dark microcosms (respectively), but no significant change in concentration in the abiotic microcosms. Likewise, there was a significant ($P<0.05$) decrease in the concentration of 1R,2R-(-)-pseudoephedrine in the biotic light microcosm only. What this showed was that even the more persistent isomers were still degraded under environmental conditions. Interestingly, the synthetic isomers were more significantly degraded in the biotic light microcosms than the biotic dark microcosms, whereas the naturally occurring isomers where more heavily degraded in the biotic dark microcosms. This suggested that different microorganisms may have been present in the biotic light and biotic dark microcosms. The changes in EF fraction were also evaluated and showed that there was a significant ($P<0.01$) decrease in the EF of (±)-ephedrine, showing enrichment with 1S,2R-(+)-ephedrine, in the biotic light and dark microcosms but no significant change ($P>0.05$) in the abiotic microcosms. Likewise, there was a significant ($P<0.01$) increase in the EF of (±)-pseudoephedrine, showing enrichment with 1R,2R-(-)-pseudoephedrine, in both of the biotic microcosms, but no change in the EF in the abiotic microcosms.

Additionally, the formation of (+)-1S, 2R-ephedrine was not accompanied by a discussion of how other anthropogenic compounds degrade in river water and wastewater, both of which are potential

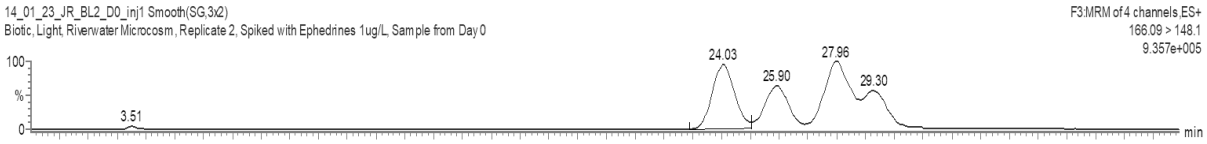
sources of the isomer. Other examples of analyte degradation in wastewater are present with the literature [26; 27] including the degradation of amphetamine-like compounds in wastewater and river water [27], which also observed differences in how compounds degraded between the two matrices and suggested it could be due to different bacterial communities. The presence of different bacterial communities could explain why (+)-1S, 2R-ephedrine was not detected in all treated wastewater and only during the second set of (-)-1R, 2S-ephedrine containing microcosms.

Lastly, whilst much is made of the environmental risk posed by (+)-1S, 2R-ephedrine due to its comparatively high stability and toxicity it is important to put this risk in context. This can be done by using risk quotients (RQs), which are used by the US environmental protection agency (EPA) to assess the ecological risk of pesticides [28]. RQ can be calculated by dividing the estimated environmental concentration of an analyte by its lowest LC_{50} or EC_{50}. As no isomer of ephedrine was detected in river water the Evans method's limits of detection were used instead to determine the maximum possible RQ, alongside the EC_{50}'s summarised in table 2. For natural and all isomer toxicity the highest limit of detection was used to generate the maximum possible RQ. The RQs in addendum table 1 showed that there was no significant risk posed by ephedrine, with even the highest RQ far below the acute toxicity level of concern, which is equivalent to an RQ of 0.5.

Addendum table 1. The risk quotient posed by ephedrine isomers to a range of river organisms

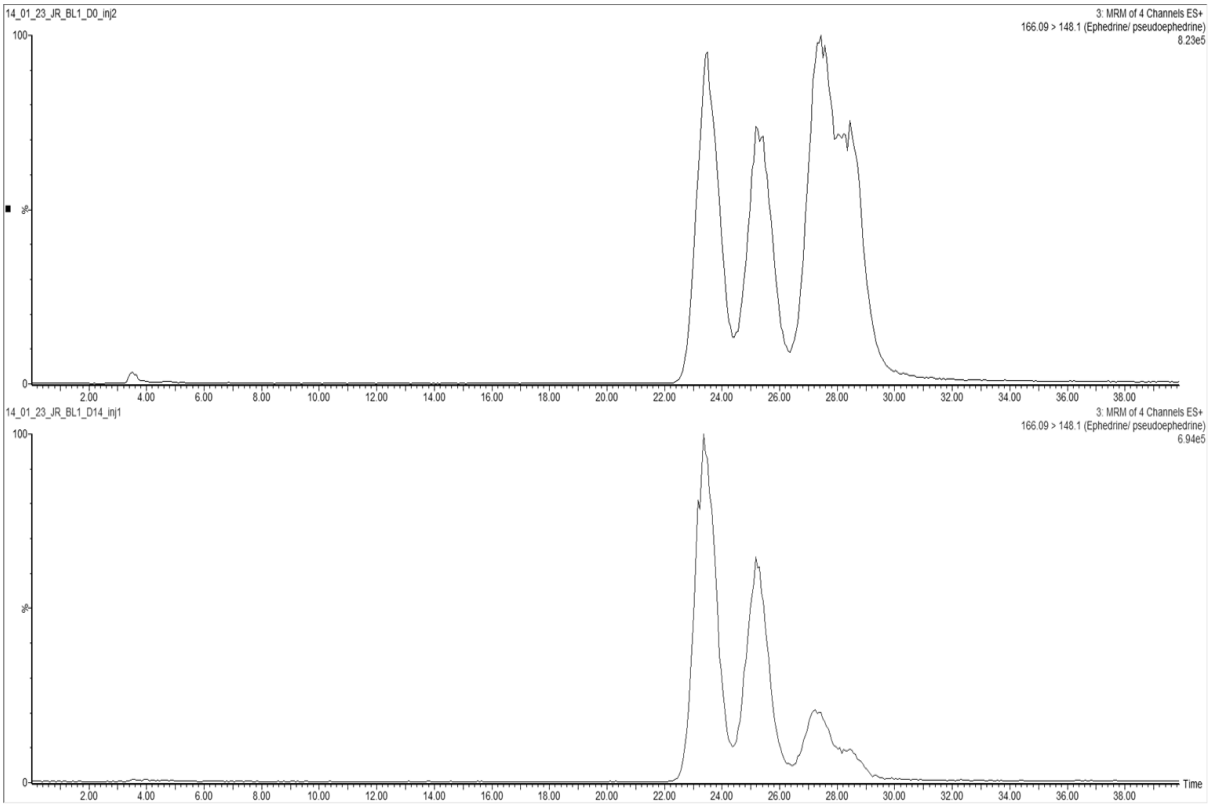
	Limit of detection ($\mu g\ L^{-1}$)	*Daphnia magna*	*Pseudokirchneriella subcapitata*	*Tetrahymena thermophila*
1S,2R-(+)-Ephedrine	0.97	5.68×10^{-6}	1.29×10^{-6}	2.28×10^{-5}
1R,2S-(-)-Ephedrine	1.83	1.71×10^{-5}	7.06×10^{-6}	5.08×10^{-5}
1S,2S-(+)-Pseudophedrine	1.46	5.32×10^{-5}	3.49×10^{-6}	1.47×10^{-5}
1R,2R-(-)-Pseudophedrine	1.38	1.29×10^{-5}	3.08×10^{-5}	3.00×10^{-4}
Mixtures				
Natural	1.83	-	-	2.96×10^{-5}
All	1.83	-	-	3.49×10^{-5}

Additionally, the chromatographic separation of the isomers was never shown in the publication. Addendum figure 1 shows a chromatogram from the mixed isomer, biotic light 1 microcosm, which was analysed via the Evans et al method. The elution order is as follows: 1S, 2R-(+)-epehdrine, 1R, 2R-(-)-pseudoephedrine, 1R, 2S-(-)-ephedrine and 1S, 2S-(+)-pseudoephedrine

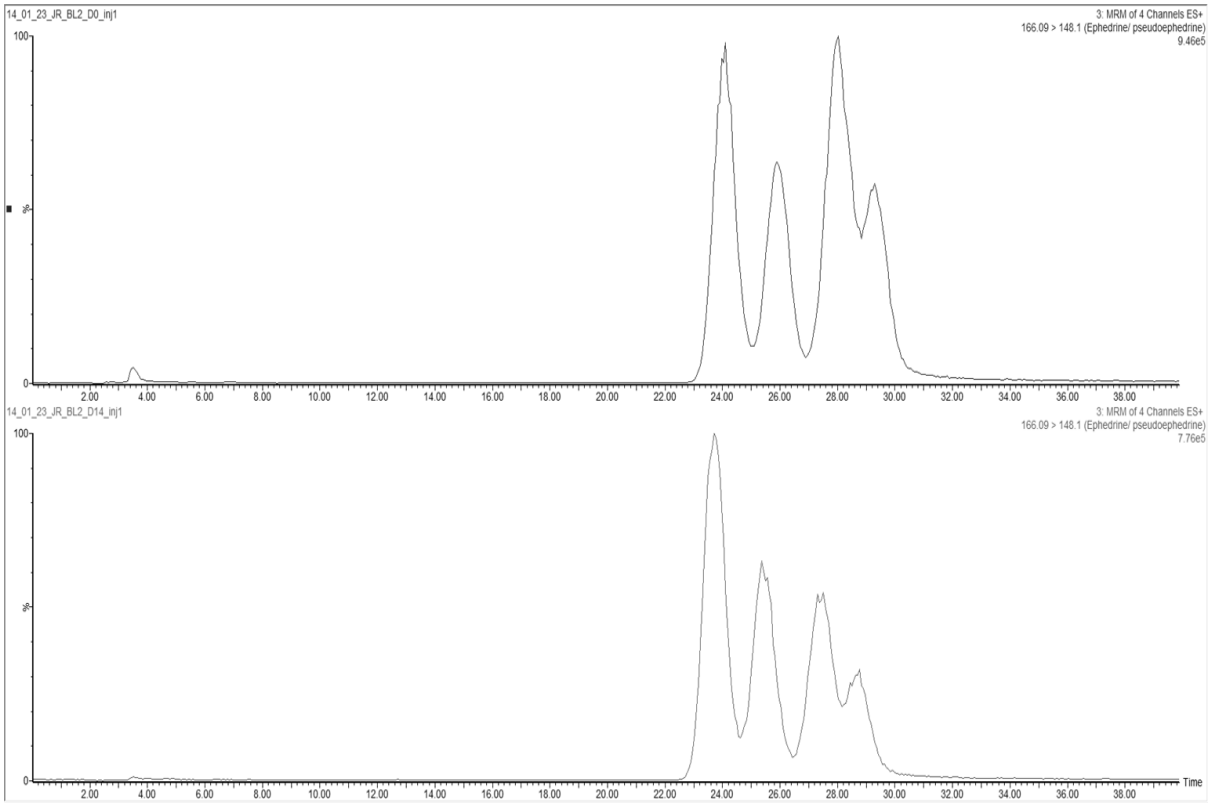


Addendum figure 1. Elution order of ephedrine in the Evans et al. method

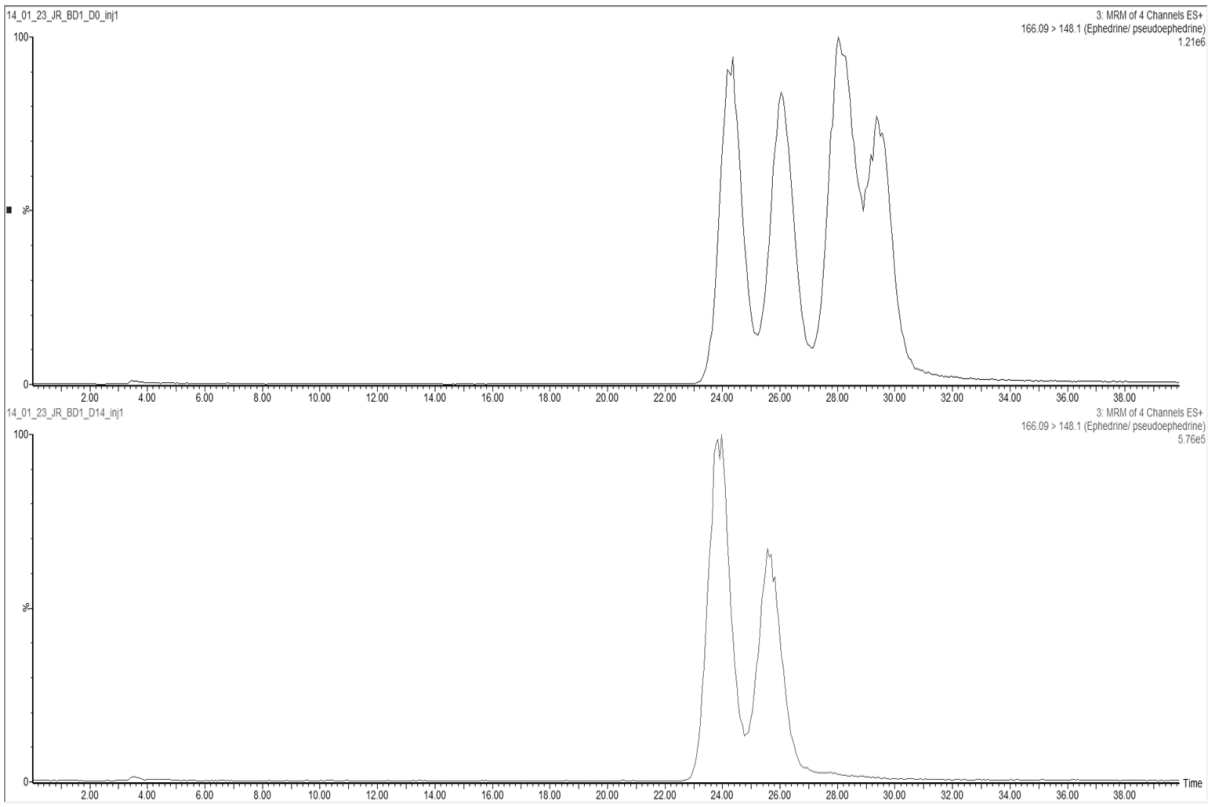
The following figures show the change in relative abundance of each isomer in the various mixed isomer microcosms on day 0 (D0) and day 14 (D14).



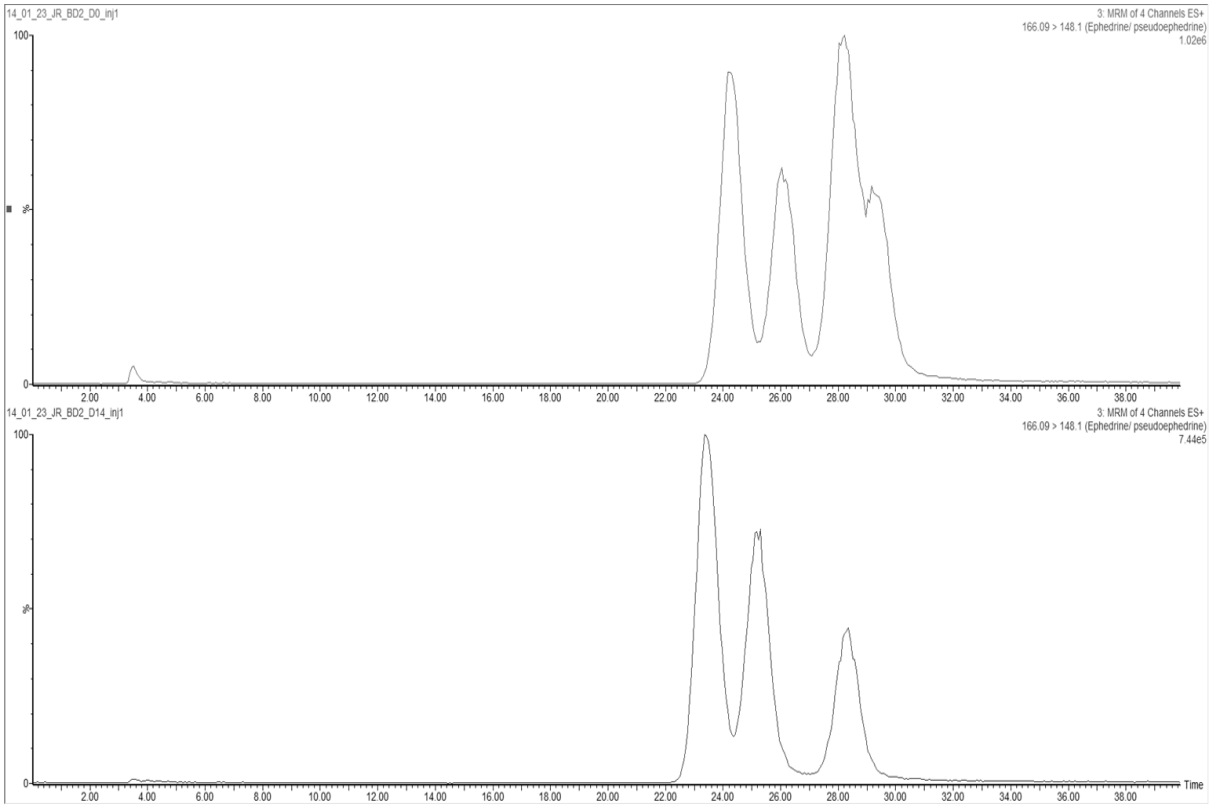
Addendum figure 2. Biotic Light microcosm one (BL1), showing decrease in the relative abundance of 1R, 2S-(-)-ephedrine and 1S, 2S-(+)-pseudoephedrine



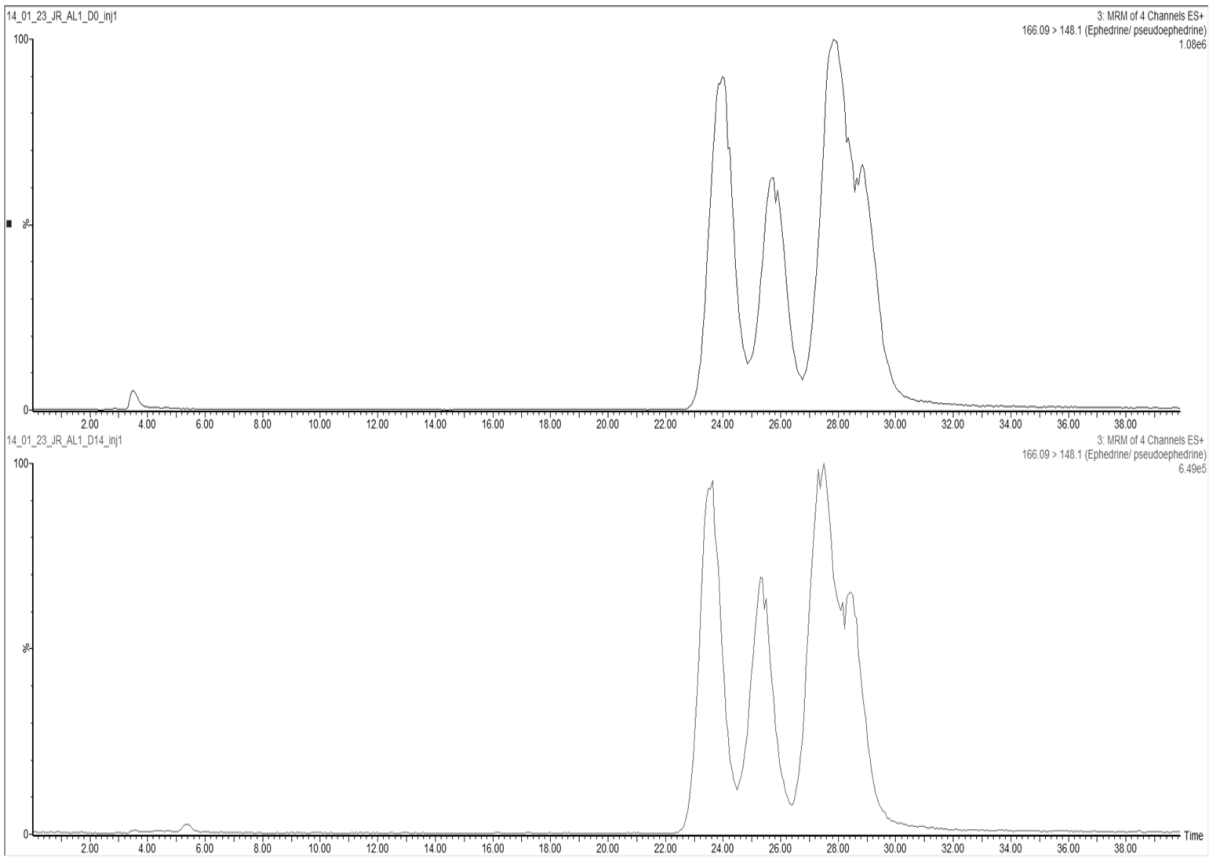
Addendum figure 3. Biotic Light microcosm two (BL2), showing decrease in the relative abundance of 1R, 2S-(-)-ephedrine and 1S, 2S-(+)-pseudoephedrine



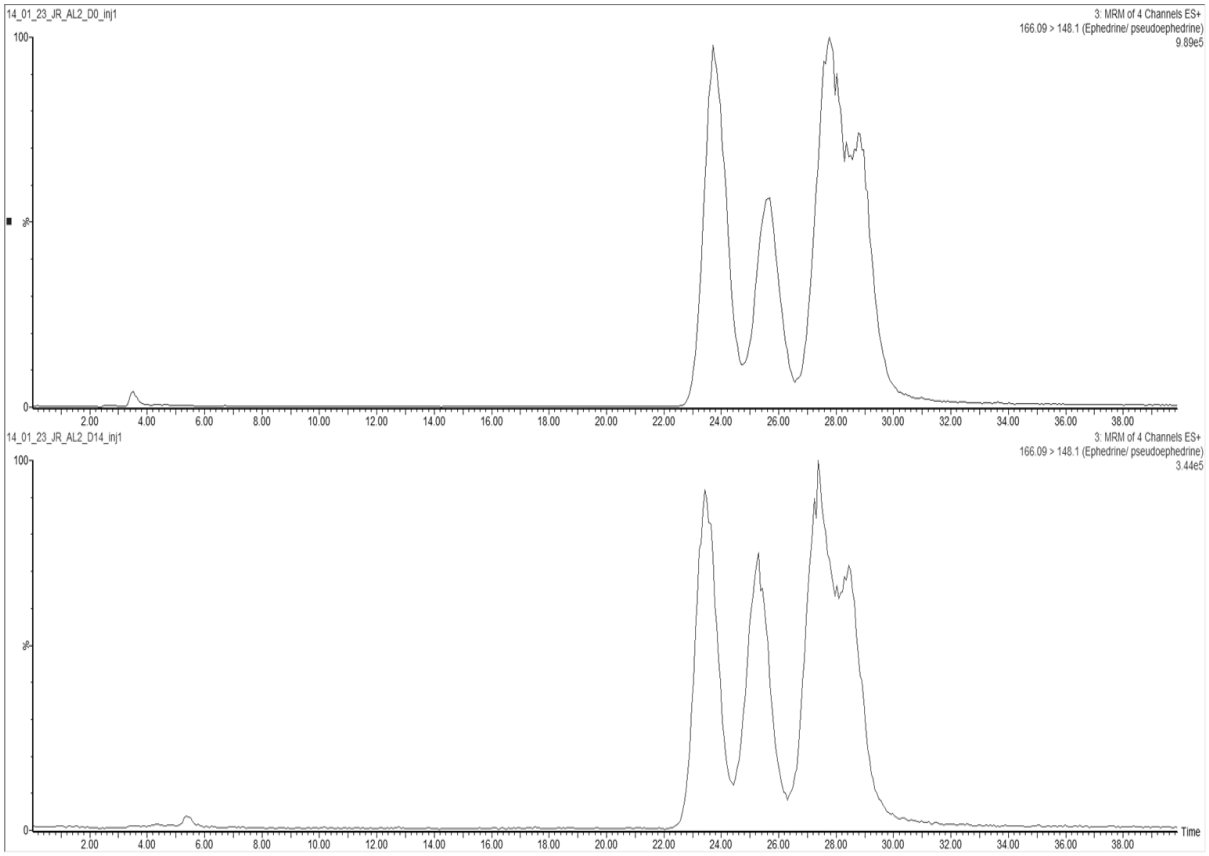
Addendum figure 4. Biotic Dark microcosm one (BD1), showing decrease in the relative abundance of 1R, 2S-(-)-ephedrine and 1S, 2S-(+)-pseudoephedrine



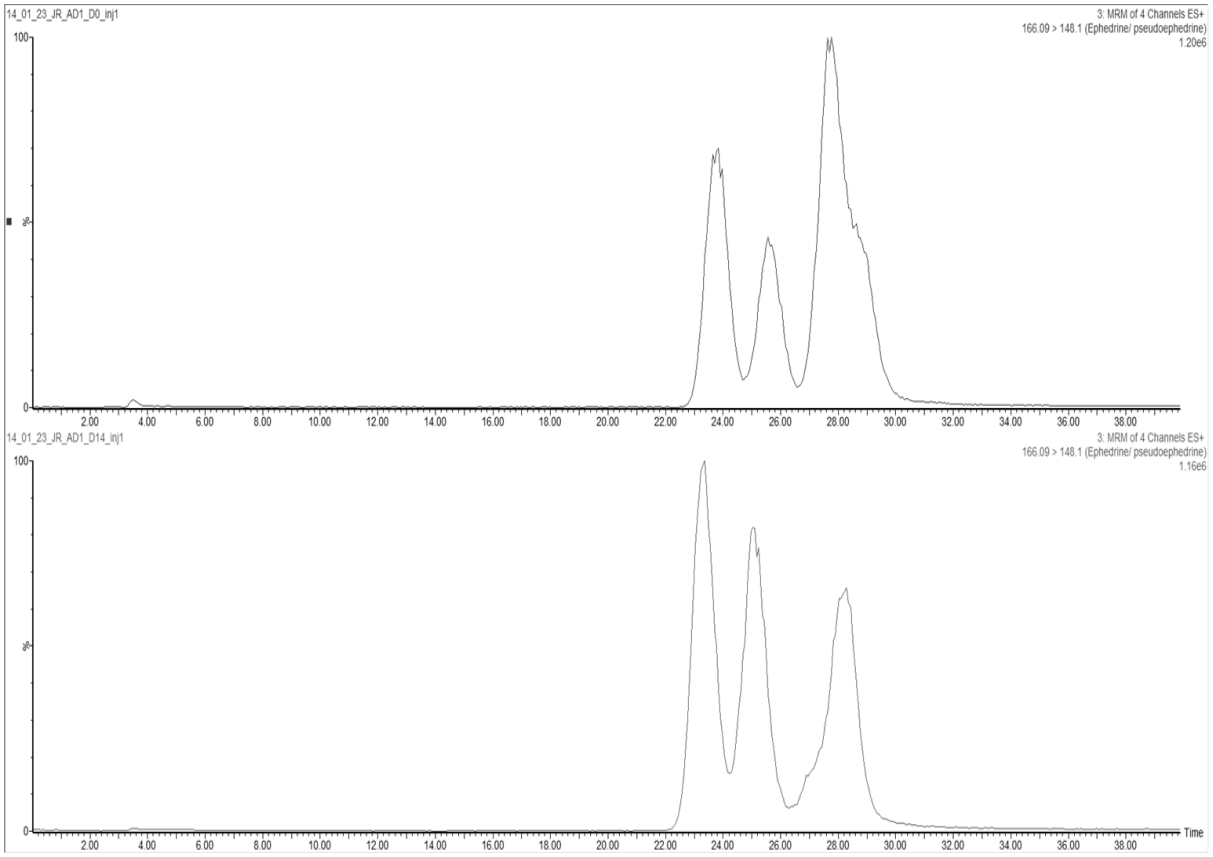
Addendum figure 5. Biotic Dark microcosm two (BL2), showing decrease in the relative abundance of 1R, 2S-(-)-ephedrine and 1S, 2S-(+)-pseudoephedrine



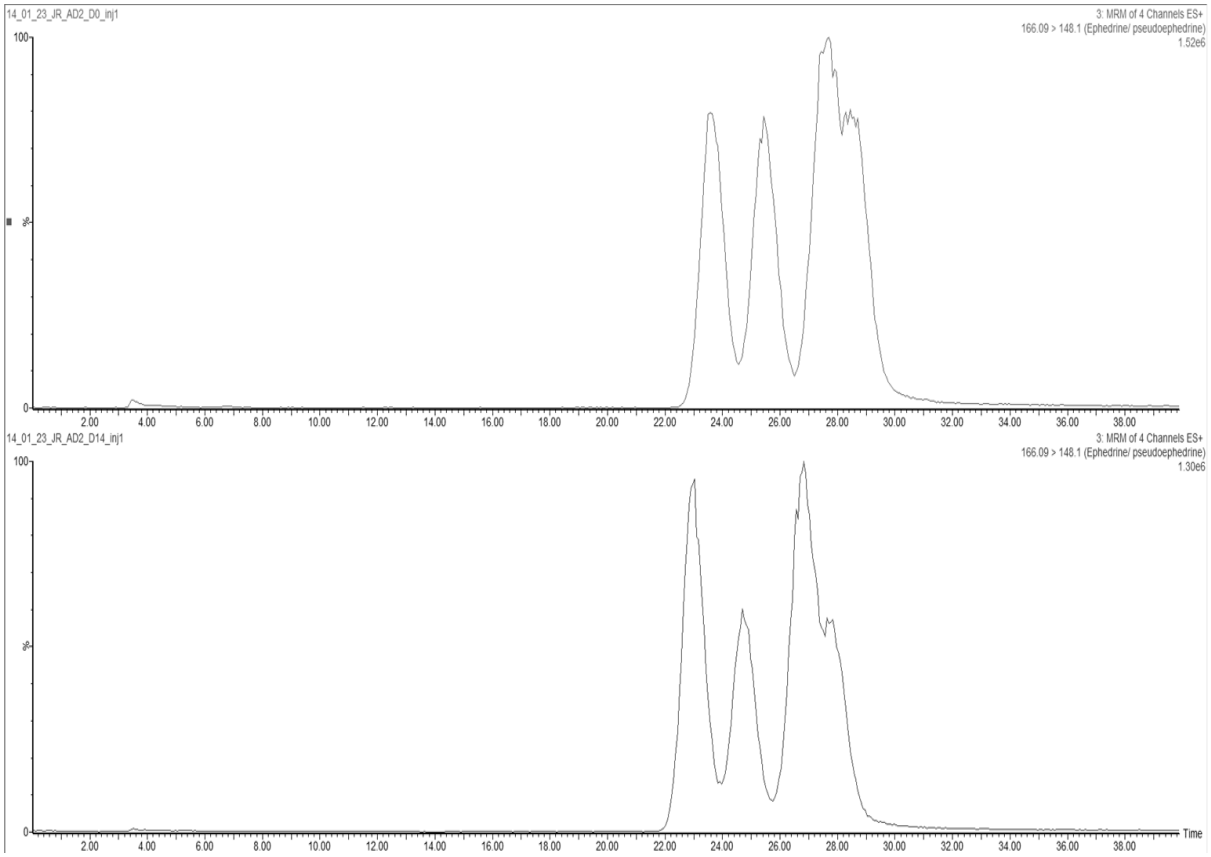
Addendum figure 6. Abiotic Light microcosm one (AL1)



Addendum figure 7. Abiotic Light microcosm two (AL2)



Addendum figure 8. Abiotic Dark microcosm one (AD1)



Addendum figure 9. Abiotic Dark microcosm two (AD2)

This declaration concerns the article entitled:								
A multi-residue supercritical fluid chromatography coupled with tandem mass spectrometry method for the analysis of chiral and non-chiral chemicals of emerging concern in environmental samples								
Publication status (tick one)								
Draft manuscript		**Submitted**		**In review**	**X**	**Accepted**		**Published**
Publication details (reference)	Rice, J., Lubben, AT., Kasprzyk-Hordern, B., 2020. A multi-residue supercritical fluid chromatography coupled with tandem mass spectrometry method for the analysis of chiral and non-chiral chemicals of emerging concern in environmental samples, *Journal of Analytical and Bioanalytical Chemistry*							
Copyright status (tick the appropriate statement)								
I hold the copyright for this material				Copyright is retained by the publisher, but I have been given permission to replicate the material here	**X**			
Candidate's contribution to the paper (provide details, and also indicate as a percentage)	The candidate contributed to / considerably contributed to / predominantly executed the… Formulation of ideas: Candidate predominantly executed the formulation of ideas around the use of SFC, analyte selection and method validation requirements in collaboration with Barbara Kasprzyk-Hordern and Anneke Lubben Design of methodology: Candidate predominantly formulated methodology in collaboration with Barbara Kasprzyk-Hordern Experimental work: Candidate predominantly executed all experimental work Presentation of data in journal format: Candidate primarily executed the writing and formatting of all data for publication, in collaboration with BKH							
Statement from Candidate	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature.							
Signed				**Date**	02/06/2020			

Chapter three introduction

In chapter one there was a focus on exploring both chirality and local prescription data for better interpretation of human health data, whilst chapter two was more explicitly focussed on exploring chirality for determining environmental toxicity. Chapter three is likewise focussed on chiral environmental analysis by developing a new analytical method using chiral supercritical fluid chromatography, but also lends itself to the analysis of public health due to its ability to analyse both CEC and WBE biomarkers in wastewater and river water.

Despite the attention on chirality in method development chapter three is primarily about developing a new method for rapidly and chirally analysing CEC in waste and environmental water. The assessment of wastewater is important for environmental health as many CECs will enter rivers from wastewater treatment. By showcasing the analytical power of SFC the chapter also seeks to make chiral analysis easier to perform, thereby, hopefully, increase the amount of research carried out using chiral chromatography and therefore increasing the amount of chiral information available to researchers assessing public and environmental health.

The initial work in the paper explores a range of different chiral analytical methodologies for analysing 140 potential CECs, including antibiotics, beta-blockers, drugs of abuse, pesticides and UV-filters. This was carried out using a single quadrupole MS instrument and was used to help establish chiral analyte separation and retention times for all analytes. For quantitative method development a triple quadrupole instrument was used instead, with the analytes divided into three groups to facilitate easier method development and sufficient analytical power. The final method was then used to analyse grab samples of influent and effluent wastewater and river water to assess the method's suitability for environmental analysis.

A multi-residue method by supercritical fluid chromatography coupled with tandem mass spectrometry method for the analysis of chiral and non-chiral chemicals of emerging concern in environmental samples

Jack Rice[a], *Anneke Lubben*[b], *Barbara Kasprzyk-Hordern*[a*]

[a] Department of Chemistry, Faculty of Science, University of Bath, Bath, BA2 7AY, United Kingdom (UK)

[b] Material and Chemical Characterisation Facility, University of Bath, Bath, BA2 7AY, United Kingdom (UK)

*****Corresponding author: Barbara Kasprzyk-Hordern, E-mail: b.kasprzyk-hordern@bath.ac.uk

Abstract

This manuscript presents the development, validation and application of a multi-residue supercritical fluid chromatography coupled with tandem mass spectrometry method for the analysis of 140 chiral and non-chiral chemicals of emerging concern in environmental samples with 81 compounds being fully quantitative, 14 semi-quantitative and 45 qualitative, validated according to European Medicine Agency (EMA) guidelines [1]. One unified LC-MS method was used to analyse all analytes, which were split into three injection methods to ensure sufficient peak resolution. The unified method provided an average of 113 % accuracy and 4.5 % precision across the analyte range. Limits of detection were in the range of 35 pg L^{-1} - 0.7 µg L^{-1}, in both river water and wastewater, with an average LOD of 33 ng L^{-1}. The method was combined with solid-phase extraction and applied in environmental samples, showing very good accuracy and precision, as well as excellent chromatographic resolution of a range of chiral enantiomers including beta blockers, benzodiazepines and antidepressants. The method resulted in quantification of 75 % of analytes in at least two matrices, and 56 % in the trio of environmental matrices of river water, effluent wastewater and influent wastewater, enabling its use in monitoring compounds of environmental concern, from their sources of origin through to their discharge into the environment.

Keywords: Supercritical fluid chromatography, environment, mass spectrometry, chiral chromatography, chemicals of emerging concern

1. Introduction

In 2000 the European Union (EU) set out its first Water Framework Directive (WFD) [1], which aimed to maintain and restore water quality across the EU by adopting a unified approach to discharge and emissions into surface waters. Thirty-three priority substances were identified and regulations were set-up to reduce their discharge into the environment. These initial priority substances were mainly metals, flame retardants and biocides. Additional compounds were prioritised by a new directive in 2012 [2]. As part of this expansion the NSAID diclofenac and the synthetic estrogens 7β-estradiol (E2) and 17α-ethinylestradiol (EE2) were proposed as potential priority substances. In 2019 the publication of the EU's *'strategic approach to pharmaceuticals in the environment*' [3] mapped out the EU's objectives for how to monitor and reduce the usage of pharmaceuticals, promote 'greener' manufacturing, improve environmental risk assessments (ERAs) for pharmaceuticals, and support monitoring of these compounds in fresh and coastal waters. However, despite this focus on pharmaceutical as chemicals of emerging concern (CECs) in the environment, there has been no assessment on the effect of chirality. The effects of chirality in pharmaceuticals for human consumption are well documented, particularly in the wake of the thalidomide disaster, however the effect of chirality on environmental toxicity is still not as well understood. Both new and existing ERAs currently do not require any examination of the effect of chirality on their environmental toxicity [4; 5]. The main reason for not acknowledging chirality in ERAs is a lack of published knowledge on the relative effects of enantiomers, as well as lack of methods enabling research in this area. New, sensitive, multi-residue methods enabling identification and quantification of chiral and non-chiral CECs within one analytical method are therefore urgently needed.

Methods have already been developed for the monitoring of CECs in surface waters [6-18] and their effect on the aquatic environment [19-21]. However, many of these methods are not performed using chiral discrimination as separation at enantiomeric level poses an analytical challenge. Enantiomers cannot be separated by conventional reversed-phase chromatography utilising C18 stationary phases. Instead specific chiral chromatography methods must be developed utilising expensive chromatography columns. The types of chiral selectors used range from small molecules like modified benzenes to macrocycles and proteins. The difficulty of chiral chromatography is that the biological nature of many chiral selectors restricts the range of solvents and other modifiers which can be used in the methods [6; 22]. This generally also restricts the ability to develop LC methods with mobile phase gradients,

thus increasing analytical runtimes. Chiral columns usually operate within inefficient HPLC modes due to large silica particle sizes being used.

Supercritical fluid chromatography (SFC) is an increasingly popular analytical technique that is of great interest for chiral analysis [23; 24]. As with other chiral chromatography, chiral-SFC requires a stationary phase containing a chiral selector, but unlike reversed-phase (RP) chiral-LC this chiral selector is a small chiral molecule, rather than a biomolecule, enabling the use of a wider range of solvents. Additionally, these columns can tolerate higher backpressures and have shorter equilibration times making the use of mobile phase gradients possible using SFC. SFC generally uses CO_2 as its primary mobile phase [25] with an organic co-solvent for elution such as n-heptane, isopropyl alcohol or methanol. This makes SFC a "greener" analytical technique compared to traditional chromatography because of the use of renewable CO_2 as a mobile phase. Acidic or basic modifiers, such as formic acid or ammonium hydroxide, are also commonly added to help limit unwanted analyte-stationary phase interactions, such as those with uncapped silanols on unmodified silica stationary phases. The main requirement for using SFC is that analytes have to be soluble in either the supercritical CO_2 or the organic co-solvent. Fortunately, most CECs are soluble in common organic solvents like methanol or acetonitrile [14; 24; 26].

(Stereoselective) analysis of trace levels of CECs is also required in wastewater-based epidemiology (WBE). Large public health monitoring studies have become increasingly wide spread, both in terms of the size of the populations studied [27-31] and the range of analytes that have been detected are often those of interest as environmental micropollutants [27; 32-40]. However, despite many of these analytes having at least one chiral centre, most published WBE analytical methods do not include enantiomer separations. Whilst sometimes desirable, this trend can be limiting as it effectively excludes analytical approaches that require long sample preparation or analysis times, like chiral HPLC. Therefore, perhaps the greatest benefit of SFC is that it could be used for the analysis of both public and environmental health determinants. Hence, this manuscript aims to deliver a rapid method with sensitive and selective multi-residue measurements of structurally variable groups of CECs.

2. Materials and Methods

2.1.Materials

High purity (≥99.97 %), food grade, gaseous CO_2 was supplied to the system from an unheated cylinder without a dip tube. All solvents, except water, were of MS-grade and purchased from VWR. Ultrapure water was obtained from a MilliQ purification system (Merck Millipore, UK). Mobile modifiers trifluroacetic acid (TFA), formic acid (FA), ammonium hydroxide, ammonium formate and ammonium acetate were all purchased from Sigma Aldrich UK. A full list of analytes and internal standards, and their associated supplier information, is provided in the supplementary materials section (Table S1). All glassware used was silanised with dimethylchlorosilane (DMDCS in toluene, Sigma-Alrich) prior to use to limit adsorption of basic analytes to silanol sites on the surface of the glassware. Solid-phase extraction cartridges used for validation were Oasis, 60 mg, 3 cc, HLB SPE cartridges (Waters, UK). Sample evaporation was performed using a turbovap LV concentration workstation (Caliper, UK). Whatman GF/F filter papers were used for all water sample filtrations.

2.2 Methods

A Waters Acquity UltraPerformance Covergence Chromatography (UPC^2) instrument coupled with either a Waters Acquity QDa single quadrupole mass detector or a Waters Xevo TQD triple quadrupole mass spectrometer was used for method development. Three Waters chromatography columns were tested: (i) Waters Trefoil 2.1x50 mm, 2.5 µm amylose based column modified with 3,5-dimethylphenylcarbamate (AMY1) Waters Trefoil 2.1x50 mm, 2.5 µm cellulose based column modified 3,5-dimethylphenylcarbamate (CEL1), (iii) Waters Trefoil 2.1x50 mm, 2.5 µm cellulose based column modified with 3-chloro-4-methylphenylcarbamate (CEL2)

2.2.1 UPC^2-MS method development

2.2.1.1. Initial screening

Initial screening used conditions outlined in the Waters chiral method development strategy for optimal path screening [41]. A range of analytes were selected for this initial screening including both chiral and achiral compounds, with a full listing provided in the supplementary materials section (Table S1). The following four methods were trialled:

(i) AMY1 (column) with A: 100 % CO_2 and B: 1:1:1 (v/v) EtOH:IPA:MeCN with 20 mM NH_4OAc

(ii) CEL1 (column) with A: 100 % CO_2, and B: 1:1 (v/v) MeOH:IPA with 0.2 % (v/v) TFA

(iii) CEL2 (column) with A: 100 % CO_2, and B: 1:1 (v/v) EtOH/MeCN with 0.2 % (v/v) TFA

(iv) AMY1 with A: 100 % CO_2, and B: 1:1 (v/v) EtOH:IPA with 0.2 % (v/v) TFA

All columns were 2.1x50 mm Trefoil chiral columns with a particle size of 2.5 µm. Initial conditions were 3 % B mobile phase at a flow rate of 1.2 mL min^{-1}, a column temperature of 40 $^{o}C \pm 5\ ^{o}C$, an automatic backpressure regulator pressure of 3,200 psi, and a sample injection volume of 2µL. All samples were dissolved in methanol. The QDa detector was set to alternating positive and negative ESI mode and collected data in both modes for a mass range of 150-650 m/z. A mobile phase gradient was applied in all methods and consisted of holding the initial 3 % B mobile phase conditions for 0.5 minutes, before increasing the % B mobile phase to 60 % B over 1.5 minutes and then holding at 60 % B for 0.5 minutes. After this, initial conditions were restored by decreasing the mobile phase B % to 3 % B over 0.5 minutes, and holding at this level for 0.5 minutes before starting the next run. The total cycle time for each sample was 3.5 minutes, with MS data collected for 3.0 minutes. The make-up solvent used was 9:1 (% v:v) MeOH:H_2O with 1 % formic acid and a flowrate of 0.45 mL min^{-1}. Analyte detection was performed using single quadrupole MS in ESI+ and ESI- scanning mode using the following conditions: ESI+ and ESI- scan conditions used a centroid data format and scanned between 120-650 m/z over 3.5 minutes with a cone voltage of 15 V. ESI+ capillary voltage was 1.5 kV, ESI- capillary voltage was 0.8 kV. The initial optimal screening path was used to identify column chemistries and mobile phases that could resulted in at least partial separation of chiral analytes, as well as the detection of both chiral and achiral analytes.

2.2.1.2. Selection of mobile phases

Following initial screening four mobile phase compositions (A-D) were selected and tested with three different columns (see Tables S2 and S3 for details). Analytes were detected using the QDa single quadrupole MS using a targeted ESI+ MS method rather than a scanning method. Using these methods the following chiral analytes were analysed at a concentration of 100 µg L^{-1}: atenolol, bisoprolol, ketamine, metoprolol, propranolol, temazepam, zolpidem, amitriptyline, MDMA, methamphetamine, amphetamine, PMA, mephedrone, venlafaxine,

desmethylvenlafaxine, cocaine, benzoylecognine, methadone, EDDP, fluoxetine, chloramphenicol and tramadol.

2.2.2 Solid-phase extraction

To enable the sensitive analysis of environmental samples a solid phase extraction (SPE) method was developed for use with influent wastewater, effluent wastewater and river water. Prior to extraction samples were homogenised by inverting their storage containers to resuspend settled sediment. 50 mL of each sample solution was then taken and 25 µL of a 2 mg L^{-1} internal standard stock solution was added. The sample was then filtered through Whatman GF/F filter paper and stored on ice before extraction. Waters Oasis HLB cartridges were used to extract samples and were conditioned according to the manufacture's guidelines, using 2 mL of methanol followed by 2 mL of ultrapure water. Samples were loaded onto the SPE cartridges under vacuum at a rate of 3 mL min^{-1} before washing with 3 mL of ultrapure water. Samples were then left to dry under vacuum for 30 minutes. Once dried, samples were eluted or directly stored at -20 ^{o}C for future analysis. Samples were eluted using 4 mL of MeOH into silanised glass tubes before being placed in a water bath at 30 ^{o}C and evaporated in a turbovap evaporator under a gentle stream of N_2. Once completely evaporated, samples were reconstituted to a final volume of 500 µL in 100 % MeOH.

2.2.3 Method performance

Validation was carried out in accordance with recommendations set by the European Medicines Agency (EMA) [42; 43]. Method validation was performed in 100 % methanol, in influent wastewater and in river water. The following parameters were evaluated: instrument and method accuracy, precision, linearity and range, limits of detection and quantification recovery, and signal suppression.

A mixture of all available analytes was prepared from stock solutions at a concentration of 2 mg L^{-1} of each analyte in methanol, and was used to create all working solutions, spiked samples and quality controls as described below. Another mixture of deuterated compounds was also prepared at the same concentration (2 mg L^{-1} of each in methanol) and is described in the text as internal standards or internal standard mixture. The internal standard mixture was used to create all working solutions, spiked samples and quality controls as described below.

2.2.3.1. UPC^2-TQD instrument method performance

The instrument linearity and concentration range over which an analyte could be detected were determined using a seventeen point calibration curve ranging from 0.0-1000 µg L^{-1} for achiral analytes and 0.0-500 µg L^{-1} for individual chiral isomers such that the concentration of the sum of both isomers covered a range of 0.0-1000 µg/L. Calibrant concentrations used are as follows, with chiral concentrations in brackets: 0 (0) µg L^{-1}, 0.01 (0.005) µg L^{-1}, 0.05 (0.025) µg L^{-1}, 0.1 (0.05) µg L^{-1}, 0.5 (0.25) µg L^{-1}, 1 (0.5) µg L^{-1}, 5 (2.5) µg L^{-1}, 10 (5) µg L^{-1}, 25 (12.5) µg L^{-1}, 50 (25) µg L^{-1}, 100 (50) µg L^{-1}, 200 (100) µg L^{-1}, 400 (200) µg L^{-1}, 600 (300) µg L^{-1}, 800 (400) µg L^{-1} and 1000 (500) µg L^{-1}. 100 µg L^{-1} of internal standard was included in each calibrant sample. Instrument linearity was determined using the R^2 of a linear line of best fit, as determined by the data analysis software used (MassLynx V4.1). The linear range was the calibrant concentration range over which the linearity was calculated. 2 µL of each calibrant sample were injected three times for the determination of linearity.

Instrument limits of detection (iLOD) and quantification (iLOQ) were determined using the calibration curve. iLOD was determined as the lowest measured calibrant concentration with an average peak signal to noise ratio (S/N) of greater than or equal to 3 (S/N ≥ 3) across three repeat calibrant injections. iLOQ was determined as the lowest measured calibrant concentration with an average S/N ≥ 10 across three repeat calibrant injections.

Accuracy was determined at three different concentrations for both chiral and achiral analytes. For achiral analytes accuracy was determined at 10, 50 and 200 µg L^{-1}, whilst for chiral analytes accuracy was determined at 5, 25 and 100 µg L^{-1}. Samples were injected in triplicate and accuracy at each concentration (x) was calculated using the following equation (eq. 1), where x is the theoretical concentration, e.g. 10 µg L^{-1}, and $x_{1\text{-}3}$ is the concentration measured in each sample.

$$Accuracy\ (\%) = \frac{x}{\text{Average}\ (x_1, x_2, x_3)} * 100 \qquad eq.1$$

Precision was determined at the same concentrations as those used for the accuracy and determined as the relative standard deviation (RSD) between triplicate injections at each concentration (eq 2). Interim or interday precision, was calculated by determining precision using the triplicate injection of freshly prepared samples on two different, non-sequential days. Repeatability was measured as the average RSD of each day's precision.

$$Intraday\ precision\ (\%) = \frac{\sigma_{x_1 - x_3}}{Average(x_1, x_2, x_3)} * 100 \qquad eq.2$$

Relative retention time (t_{rel}) was measured as the difference between the analyte's peak retention time (t_A) in mobile phase and the peak retention time of its assigned internal standard (t_{ISTD}) (eq. 3).

$$t_{rel} = \frac{t_A}{t_{ISTD}} \qquad eq.3$$

Enantiomeric resolution (R_s) was also calculated (eq. 4). The base peak width (w_x S^{-1}) for each enantiomer was calculated as the difference between the average peak-end and peak-start times. t_n refers to the peak top retention time of the peak, with the subscript referring to the order in which the peaks eluted in.

$$R_s = \frac{t_2 - t_1}{0.5(w_2 + w_1)} \qquad eq.4$$

Enantiomeric fraction (EF) was measured as the relative concentration of the first eluting enantiomer (E_1) relative to the sum of the concentration of both enantiomers (eq. 5)

$$EF = \frac{[E_1]}{[E_1] + [E_2]} \qquad eq.5$$

2.2.3.2. SPE-UPC^2-TQD validation

Signal suppression (SS) was calculated by comparing analyte peak areas in river water (RW) or wastewater (WW) matrix to equivalent peak areas in mobile phase (eq. 6). Samples of each matrix underwent filtration and SPE as described above, but without addition of internal standards. After matrix elution, 50 ng of each internal standard and 50 ng of each analyte were added.

$$Signal\ Suppression\ = 1 - \left(\frac{Analyte\ peak\ area\ \frac{RW}{WW}_{SS} - Analyte\ peak\ area\ \frac{RW}{WW}_{Blank}}{Analyte\ peak\ area\ QC_{100}}\right) * 100 \qquad eq\ 6$$

Absolute and relative recoveries were calculated by comparison of analyte peak areas or concentrations in river water (RW) or influent wastewater (WW), to analyte peak areas or concentrations in mobile phase (eq. 7). Both recoveries were determined in triplicate at three different concentrations, and then averaged. Analyte was spiked into samples of matrix to give a concentration of 0, 0.11, 0.5 or 2 µg L^{-1}, along with 50 ng of each internal standard, before filtration and SPE as described above (2.2.1.4). Analyte concentrations were calculated using calibration curves prepared as outlined above (2.2.2.1), and internal standards assigned by using a combination of similar retention times ($t_{rel} \approx 1$) and signal suppression ($SS_{analyte} \approx SS_{ISTD}$) factors.

$$Absolute\ recovery\ (\%) = \frac{Analyte\ peak\ area\ RW/WW_x - Analyte\ peak\ area\ RW/WW_0}{Analyte\ peak\ area\ QC_x} * 100\ eq.7$$

Method accuracy and precision were calculated at 0.05, 0.5 and 2 µg L^{-1} by spiking each analyte into either RW or WW at these concentrations. Method accuracy was calculated (eq. 8) to determine how close the measured concentration (Analyte conc. $RW/WW_{x1\text{-}x3}$) was to spiked concentrations (x), whilst method precision (eq. 9) was used to measure how similar the measured concentrations values were to each other. For each equation the concentration of analyte in the blank RW or WW samples (Analyte conc. $RW/WW_0)_{x1\text{-}x3}$ was subtracted from the measured concentration, to account for analyte already present in the matrix. The standard deviation of RW/WW concentration is denoted by σ.

$$Method\ accuracy\ (\%) = \frac{x}{Average\ (Analyte\ conc.RW/WW_x - Analyte\ conc\ RW/WW_0)_{x1-x3}} * 100\ eq.8$$

$$Method\ precision\ (\%\ RSD) = \frac{\sigma(Analyte\ conc.RW/WW_x - Analyte\ conc\ RW/WW_0)_{x1-x3}}{Average\ (Analyte\ conc.RW/WW_x - Analyte\ conc\ RW/WW_0)_{x1-x3}} * 100\ eq.9$$

Enantiomeric fraction and chiral peak resolution were calculated at 5, 50 and 200 µg L^{-1} using eq. 4 and eq. 5 respectively, in both river water and wastewater.

Method LOD (mLOD) and method LOQ (mLOQ) were calculated using the instrument LOD (iLOD) and instrument LOQ (iLOQ) calculated in 2.2.2.2 and the average relative recovery ($Rec_{average}$) calculated above (2.2.2.8) (eq. 10). mLOQ was also calculated using eq. 10 by substituting iLOD for iLOQ. CF is the concentration factor of the SPE method described above (2.2.1.4), which is calculated as the initial volume of matrix used (50 mL) divided by the final sample volume (0.5 mL).

$$mLOD = \frac{iLOD * 100}{Rec_{Average} * CF} \quad eq.10$$

2.2.4. Analysis of environmental samples

Samples of influent, effluent and river water were collected by grab sampling to test the suitability of the method in the analysis of real samples. Influent and effluent grab samples were collected on the same day, at the same time, from a wastewater treatment plant (WWTP) serving a city in the South-West of the UK which discharges its effluent into a river. River water grab samples were collected mid-stream, upstream and downstream of the

WWTP where influent and effluent samples were also collected. All samples were transported back to the lab on ice in separate HDPE containers, and prepared as described in 2.2.1.4. Two samples were prepared for each matrix and each sample was injected and analysed in triplicate. Enantiomeric fractions of chiral analytes were calculated using eq. 5. Two rounds of samples were collected. The first set of environmental samples were collected in early January 2018 and were used for method validation, as detailed above, whilst the second set were collected in February 2018 and analysed for as proof of concept (3.3).

3. Results and discussion

3.1 Method development

3.1.1. Initial screening

Results from the initial screening using conditions described in 2.2.1 can be generalised as follows: (1) Amphetamine and MDMA's enantiomers were not separated by any of the four methods. Ketamine was partially or fully separated by all but the CEL-1 method. (2) CEL-1 was the only method to partially separate methamphetamine and mephedrone. (3) CEL-2 was able to only partially separate venlafaxine and ketamine. (4) No combination of methods was able to separate all chiral analytes

3.1.2. Selection of mobile phase composition

Table S4 shows the enantioselective separation that was achieved when using the conditions described in Tables S2 and S3 to separate a range of chiral pharmaceuticals and drugs of abuse. The most consistent and best performing method was method B2, using CEL-1 and the following mobile phase composition: mobile phase A: CO_2, mobile phase B: 1:1:1 (v/v) MeOH:MeCN:IPA at a flow rate of 1.5 mL min^{-1} with a total runtime of 9 minutes. Mass chromatograms showing the enantioselective separation are presented in Figure S1. This method was considered as performing best and was therefore selected for validation. The analytical set-up was altered to allow for coupling the SFC to the triple quadrupole instrument. This was achieved by installing a splitter on the SFC instrument, post-dilution with make-up solvent, channelling the flow into the switching valve of the adjoining Xevo TQD instrument instead of into the QDa module of the UPC^2. This transition to the new instrument also necessitated adaptations to the mobile phase conditions due to higher system backpressures. The mobile phase flow rate was therefore decreased to 0.75 mL min^{-1} with all other chromatography conditions left unaltered. The MS conditions used were as follows: capillary voltage 3.0 kV, desolvation temperature 400.0 ^{o}C, source temperature 150.0 ^{o}C,

cone gas flow 100.0 L min^{-1}. The MRM transitions, cone voltages and collision energies used with the TQD instrument are detailed in Table S5. In total 210 compounds were analysed using method B2, necessitating their division into multiple MS methods. First the analytes and deuterated internal standards ionised under negative ESI conditions were selected for inclusion in the NEG method. The remaining analytes were then separated into two groups to create a total of three methods, all with a similar number of scan points per analyte peak. The methods can be summarised as follows: (i) DAC method: drugs of abuse, antibiotics and chiral analytes, and associated deuterated internal standards ionised in ESI+ mode, (ii) PHARMA method: pharmaceuticals, pesticides and other analytes, and associated internal standards ionised in ESI+, (iii) NEG method: analytes ionised in ESI- (Table S6).

3.2 Method validation

3.2.1 UPC^2-TQD validation

Instrument linearity, limits of detection and quantification are shown in Table S7. Internal standards were assigned using analyte and internal standard retention time, signal suppression and absolute recovery factors, which are shown in the supplementary material (Table S8), with priority given to (in order) relative retention time and analyte signal suppression in wastewater and river water. EMA guidelines were used to determine which compounds were fully quantitative, with compounds that did not meet the required specifications being described as semi-quantitative or qualitative, using criteria discussed below.

Linearity results were generally excellent with most analytes showing a calibration R^2 value >0.997, with only fexofenadine, iopromide, O-desmethyl naproxen and triclosan being considered as semi-quantitative due to an R^2 between 0.990 and 0.997. Semi-quantitative compounds appear in italics in Table S7. The linearity results were used to create a calibration curve to quantify each of the analytes relative to its assigned internal standard. Instrument accuracy and precision were then measured on three non-consecutive days over the course of a week, with new samples prepared each day. Results of average instrument accuracy and precision determinations at three concentrations are shown in Table 9.

Accuracy at each concentration should be 100 % ± 20 % and where results are outside of this limit they were recorded in italics in Table S9. This deviation largely occurred in the 10 µg L^{-1} samples. Compounds in italics are considered to be semi-quantitative. Precision was recorded as required < 20 % RSD for most analytes. Precision of >20 % occurred mostly in analytes at 10 µg L^{-1}. The instrument's ability to resolve enantiomers was assessed along with

the enantiomeric fraction in mobile phase, which should be close to 0.5. Resolution and EF were calculated at 10, 50 and 200 µg L^{-1} and the average results are shown in Table 1. The resolutions are all greater than 1.2 and therefore sufficient for quantification [44; 45]. E2-tramadol was used to calculate resolution but not EF as it could not be successfully validated. Figures S2-4 show the extracted ion chromatograms obtained for each analyte in mobile phase at a concentration of 100 µg L^{-1} using the MRM1 transitions detailed in Table S5. Figures S2-4 were broken down alphabetically by MS method used, as described in Table S6.

Table 1. Enantiomeric fraction and peak resolution for chiral analytes (n=12)

Analytes	Mobile phase			
	EF	**SD**	**R_s**	**SD**
10,11-dihydro-10-hydroxy-carbamazepine	0.5	0.02	10.5	0.8
Alprenolol	0.5	0.01	5.6	0.1
Atenolol	0.5	0.02	26.3	2.5
Bisoprolol	0.5	0.02	12.2	3.3
Metoprolol	0.5	0.01	12.6	2.5
Mirtazapine	0.5	0.01	8.7	0.2
Oxazepam	0.5	0.02	15.5	2.1
Propanolol	0.5	0.07	18.2	3.3
Tramadol	-	-	8.6	1.6

3.2.2 SPE-UPC^2-TQD validation

Average relative SPE-UPC^2-TQD method recovery was determined at three concentrations (0.1, 0.5 and 2 µg L^{-1}) and presented in Table 2 as averages. Full recoveries at each concentration are presented elsewhere (Table S8 and S10). Signal suppression was calculated at 1µg L^{-1} only.

Table 2. Average relative method recovery at three concentrations (n=9) and signal suppression (n=3) in river water and wastewater (semiquantitative compounds are presented in italics)

Analyte	Average relative recovery				Signal Suppression (%)			
	River water		Wastewater		River water		Wastewater	
	%	**SD**	**%**	**SD**	**%**	**SD**	**%**	**SD**
Aminorex	58	10.2	120	9.4	12	0.07	-23	0.07
Anhydroecgonine methylester	97	5.9	108	1.1	-17	0.05	-14	0.05
Benzophenone-1	60	9.3	102	18.6	-42	0.12	-82	0.11
Benzophenone-4	97	3.6	89	2.3	-51	0.23	-21	0.16
Benzoylecgonine	101	10.6	88	16.9	-3	0.02	-2	0.02
Benzylpiperizine	*37*	*8.9*	*49*	*2.2*	*46*	*0.04*	*33*	*0.03*
Bezafibrate	66	12.5	69	7.1	-17	0.05	-17	0.04
Buprenorphine	53	10.6	61	5.2	1	0.12	30	0.12
Candesartan Cilexetil	*47*	*19.0*	*71*	*16.6*	*-4*	*0.04*	*-82*	*0.06*
Carbamazepine	90	9.9	89	6.8	-1	0.03	1	0.03
Carbamazepine 10,11 epoxide	86	12.7	90	18.8	-2	0.03	-18	0.04
Carprofen	*62*	*0.9*	*47*	*8.2*	*-10*	*0.00*	*2*	*0.00*

Citalopram	83	8.2	57	11.8	4	0.05	18	0.05
Clothiniadin	79	13.5	104	10.4	-11	0.04	-30	0.03
Cocaethylene	90	6.6	95	1.6	-9	0.01	-3	0.02
Cocaine	89	0.8	90	3.1	-3	0.02	4	0.02
Codeine	118	30.0	68	15.6	-3	0.06	16	0.08
Cotinine	109	10.9	77	11.7	-2	0.03	10	0.05
Desmethylcitalopram	76	15.0	125	15.4	35	0.03	-60	0.04
DHMA	*82*	*4.2*	*54*	*7.4*	*17*	*0.07*	*50*	*0.12*
Diazepam	84	9.1	93	2.9	-4	0.05	-4	0.03
Diazinon	65	11.5	122	15.1	-10	0.06	-82	0.12
Diclofenac	82	2.4	75	14.7	-28	0.02	-24	0.04
Dihydrocodeine	87	2.6	81	3.4	-1	0.02	7	0.08
Dihydroketoprofen	86	11.7	57	18.1	-16	0.11	-2	0.08
Dihydromorphine	90	3.4	88	17.5	4	0.04	18	0.03
Diltiazem	*75*	*6.5*	*68*	*30.6*	*21*	*0.01*	*55*	*0.02*
Duloxetine	*24*	*3.6*	*49*	*5.1*	*48*	*0.04*	*11*	*0.05*
E1-10,11-dihydro-10-hydroxycarbamazepine	111	14.3	112	13.7	-12	0.01	1	0.04
E1-Alprenolol	83	13.8	66	5.0	2	0.05	33	0.05
E1-Atenolol	93	7.3	95	1.4	-5	0.03	4	0.04
E1-Bisoprolol	87	10.1	89	18.1	-7	0.03	-7	0.02
E1-Metoprolol	87	11.3	80	14.7	-6	0.05	1	0.05
E1-Mirtazapine	80	8.1	78	2.8	2	0.05	15	0.05
E1-Oxazepam	85	7.0	79	12.8	2	0.06	8	0.04
E1-Propanolol	89	6.9	85	10.0	-2	0.03	-6	0.04
E1-Tramadol	*89*	*19.9*	*37*	*11.1*	*3*	*0.06*	*55*	*0.04*
E2-10,11-dihydro-10-hydroxycarbamazepine	101	5.6	113	7.2	-8	0.03	-4	0.02
E2-Alprenolol	69	11.8	72	1.9	5	0.09	19	0.07
E2-Atenolol	88	9.8	95	1.1	-5	0.03	4	0.03
E2-Bisoprolol	86	9.5	90	3.5	-1	0.04	-1	0.04
E2-Metoprolol	82	14.6	93	6.5	5	0.03	2	0.03
E2-Mirtazapine	80	10.3	80	0.2	1	0.02	9	0.02
E2-Oxazepam	94	5.0	79	9.4	4	0.06	6	0.05
E2-Propanolol	93	2.4	87	1.1	2	0.06	2	0.06
Ethylparaben	91	11.6	87	4.5	-38	0.08	-59	0.07
Fexofenadine	*89*	*8.4*	*50*	*15.1*	*-1*	*0.06*	*21*	*0.08*
Griseofulvin	88	5.0	84	11.0	4	0.05	22	0.04
Heroin	88	3.9	87	2.3	-7	0.04	-7	0.06
HMA	59	5.4	77	3.8	15	0.02	-6	0.02
HMMA	*81*	*8.8*	*147*	*12.1*	*-3*	*0.04*	*-52*	*0.04*
Hydrocodone	94	6.6	74	8.1	8	0.01	11	0.04
Imatinib	68	5.8	100	19.1	-9	0.02	-42	0.02
Imidacloprid	104	2.9	137	17.1	-24	0.03	-63	0.02
Indoprofen	79	10.9	70	4.9	4	0.01	24	0.03
Iopromide	*88*	*10.1*	*163*	*13.3*	*15*	*0.05*	*-139*	*0.05*
Ketamine	92	17.3	104	7.2	-15	0.04	-11	0.04
Ketoprofen	94	29.2	68	19.2	-86	0.17	-85	0.16
MDA	70	9.5	89	14.0	-9	0.02	3	0.03
MDMA	81	10.2	82	11.2	-4	0.04	4	0.04
MDPV	75	9.5	87	4.0	-2	0.04	-1	0.03
Memantine	*95*	*19.3*	*161*	*9.3*	*-14*	*0.05*	*-19*	*0.04*
Mephedrone	76	14.9	62	8.3	14	0.04	38	0.03
Metazachlor	96	4.4	83	14.0	14	0.05	13	0.05
Methadone	79	9.8	78	4.8	-6	0.07	4	0.05
Methamphetamine	80	0.4	90	5.2	9	0.04	13	0.04
Methylparaben	99	0.9	102	1.6	-63	0.11	-73	0.11
Morphine	98	3.1	102	6.9	1	0.06	11	0.08

Nordiazepam	84	7.4	96	4.0	-9	0.03	-6	0.03
Norephedrine	82	6.1	72	9.4	1	0.02	-13	0.03
Normorphine	51	6.9	66	5.6	32	0.04	29	0.04
Nortriptyline	62	1.7	71	13.2	7	0.06	3	0.05
O-Desmethylnaproxen	72	11.0	88	33.9	-23	0.00	-50	0.00
Omeprazole	95	11.8	132	13.2	1	0.04	-48	0.06
Oxadiazon	65	16.8	74	18.9	22	0.06	0	0.07
Oxycodone	91	4.2	104	13.7	-3	0.03	4	0.03
Oxymorphone	*13*	*3.6*	*45*	*6.8*	*81*	*0.05*	*52*	*0.05*
Pholcodine	90	3.4	109	8.9	-18	0.04	-39	0.12
Praziquantrel	85	9.9	96	5.5	-3	0.07	-2	0.04
Propylparaben	73	35.5	88	22.5	-81	0.07	-113	0.06
Quetiapine	82	4.9	106	23.5	-1	0.03	-2	0.02
Risperidone	81	5.0	93	5.4	6	0.03	-10	0.04
Salbutamol	91	9.8	94	8.5	-5	0.02	-48	0.04
Sotalol	78	9.2	146	3.7	10	0.02	-83	0.04
Sulphadiazine	57	7.3	70	5.6	12	0.04	13	0.05
Sulphamethoxazole	119	14.2	119	1.8	17	0.04	-12	0.04
Sulphapyridine	87	6.8	111	9.9	17	0.04	9	0.04
Terbutaline	99	0.2	84	1.5	99	0.01	12	0.01
Terbuthylazine	75	5.0	87	9.6	13	0.06	10	0.07
Tetramisole	96	6.8	93	0.2	3	0.05	5	0.07
Thiamethoxam	*113*	*6.3*	*149*	*15.4*	*-40*	*0.03*	*-58*	*0.04*
Triclosan	*90*	*38.6*	*36*	*5.1*	*-59*	*0.00*	*-187*	*0.00*
Valsartan	80	4.2	74	3.5	-16	0.05	-8	0.04
Vardenafil	83	4.2	103	4.6	10	0.01	-27	0.03
Zolpidem	87	6.7	112	20.6	-7	0.05	-34	0.07

Average relative recovery results were considered fully-quantitative if within 80-120 %, although compounds with lower accuracies were accepted as fully quantitative provided the average SD was <20 %. Semi-quantitative compounds again appear in italics. Briefly, 66 analytes in river water and 52 analytes in influent wastewater showed relative recovery at 80-120 %. 29 compounds in river water and 43 compounds in influent wastewater had recoveries <80 % or >120 % (Table 2). Signal suppression should ideally be close to zero with a negative signal suppression indicating signal enhancement of the analytes in matrix. The results are presented in Table 2. Most compounds were observed to have signal suppression of <20 % and signal enhancement of <20 %. Exceptions include: oxymorphone and terbutaline with high signal suppression exceeding 80 %, and ketoprofen and propylparaben with high signal enhancement exceeding 80 % in river water. In influent wastewater no analytes had signal suppression exceeding 80 %, but eight analytes (benzophenone-1, candesartan cilexetil, diazinon, iopromide, ketoprofen, propylparaben, sotalol and triclosan) had high signal enhancement of >80 %.

Method recovery values were used to calculate method limits of detection and quantification, which are presented by matrix in Tables 3 and 4. Most analytes had mLOD of <33 ng L^{-1} and

mLOQ of <78 ng L^{-1}, exceptions include benzophenone-4, ethylparaben, ketoprofen and sotalol.

Table 3. Method limits of detection, quantification, method accuracy and method precision in river water (n=9) (semiquantitative compounds are presented in italics)

Analyte	River water					
	mLOD (µg L^{-1})	mLOQ (µg L^{-1})	Average Accuracy		Average Precision	
			%	SD	%	SD
Aminorex	0.009	0.02	113.4	34.8	3.5	2.4
Anhydroecgonine methylester	0.0004	0.0007	130.3	22.4	1.4	0.9
Benzophenone-1	0.01	0.06	110.8	25.5	9.2	0.6
Benzophenone-4	0.2	0.4	99.0	9.5	11.7	9.6
Benzoylecgonine	0.0001	0.0006	110.3	18.1	2.2	2.1
Benzylpiperizine	*0.004*	*0.02*	*89.8*	*2.4*	*3.6*	*2.9*
Bezafibrate	0.01	0.06	105.9	18.5	12.6	3.8
Buprenorphine	0.02	0.09	12.2	3.6	17.4	8.7
Candesartan Cilexetil	*0.0002*	*0.001*	*2393.5*	*1226.7*	*3.5*	*0.7*
Carbamazepine	0.001	0.007	119.1	13.4	1.3	0.4
Carbamazepine 10,11 epoxide	0.001	0.006	106.1	16.6	4.6	3.6
Carprofen	*0.1*	*0.3*	*83.5*	*0.3*	*4.8*	*3.5*
Citalopram	0.002	0.008	112.3	42.3	3.1	1.3
Clothiniadin	0.0007	0.001	135.9	17.4	3.3	1.2
Cocaethylene	0.0006	0.001	122.8	21.7	3.2	0.6
Cocaine	0.0007	0.001	111.2	16.2	1.9	0.6
Codeine	0.0007	0.001	111.6	9.9	5.8	3.0
Cotinine	n/a	n/a	97.0	21.4	1.2	1.0
Desmethylcitalopram	0.02	0.08	1587.2	1012.6	0.7	0.4
DHMA	*0.1*	*0.2*	*108.8*	*1.5*	*21.2*	*24.0*
Diazepam	0.01	0.02	123.5	15.3	5.5	5.8
Diazinon	0.0007	0.001	663.3	226.3	4.4	1.6
Diclofenac	0.01	0.05	103.3	2.1	12.6	2.6
Dihydrocodeine	0.01	0.07	111.5	20.0	3.0	1.8
Dihydroketoprofen	0.1	0.3	81.7	13.6	20.9	2.3
Dihydromorphine	0.002	0.01	76.2	7.2	3.6	0.7
Diltiazem	*0.003*	*0.006*	*642.5*	*374.1*	*0.6*	*0.5*
Duloxetine	*0.0006*	*0.003*	*810.0*	*299.2*	*6.9*	*3.6*
E1-10,11-dihydro-10-hydroxycarbamazepine	0.07	0.2	110.3	19.8	5.8	3.2
E1-Alprenolol	0.0007	0.003	87.4	18.1	5.7	2.5
E1-Atenolol	0.003	0.006	115.4	11.0	5.9	2.3
E1-Bisoprolol	0.0007	0.004	78.9	15.8	3.2	2.4
E1-Metoprolol	0.003	0.0006	78.8	13.9	3.0	0.3
E1-Mirtazapine	0.0001	0.007	124.3	19.0	2.6	1.1
E1-Oxazepam	0.0007	0.003	94.7	8.6	8.0	2.5
E1-Propanolol	0.007	0.01	87.2	14.2	4.3	2.9
E1-Tramadol	*0.003*	*0.006*	*95.1*	*18.5*	*5.4*	*1.5*
E2-10,11-dihydro-10-hydroxycarbamazepine	0.006	0.01	122.5	17.6	5.2	4.5
E2-Alprenolol	0.0006	0.003	107.6	20.4	8.7	7.2
E2-Atenolol	0.003	0.006	115.6	13.9	5.4	3.1
E2-Bisoprolol	0.003	0.007	100.9	19.1	2.9	0.8
E2-Metoprolol	0.01	0.07	84.2	9.9	5.1	4.7
E2-Mirtazapine	0.0002	0.008	128.7	18.0	3.4	1.1
E2-Oxazepam	0.0005	0.001	87.5	17.5	6.2	1.5
E2-Propanolol	0.004	0.07	87.5	17.6	4.7	2.5
Ethylparaben	0.7	1	101.6	18.8	2.2	0.7

Fexofenadine	*0.2*	*0.4*	*62.8*	*9.8*	*9.0*	*2.6*
Griseofulvin	0.008	0.02	108.1	16.1	5.2	1.5
Heroin	0.002	0.009	110.8	27.4	4.3	1.1
HMA	0.02	0.03	110.7	14.6	4.9	3.7
HMMA	*0.0007*	*0.001*	*115.0*	*27.4*	*4.1*	*1.2*
Hydrocodone	0.0009	0.002	97.2	36.4	2.2	1.7
Imatinib	0.007	0.01	1695.3	601.5	1.3	0.4
Imidacloprid	0.002	0.003	74.3	16.7	2.3	0.8
Indoprofen	0.001	0.007	88.2	9.9	5.0	2.1
Iopromide	*0.002*	*0.009*	*222.9*	*114.9*	*3.2*	*2.5*
Ketamine	0.001	0.006	121.5	20.2	2.7	1.2
Ketoprofen	0.3	0.7	121.3	41.0	14.5	0.1
MDA	0.001	0.007	135.8	15.1	2.1	0.3
MDMA	0.001	0.006	87.1	15.6	2.0	0.3
MDPV	0.0001	0.0007	84.7	6.3	3.0	0.9
Memantine	*0.007*	*0.01*	*184.4*	*61.2*	*9.3*	*7.6*
Mephedrone	0.0001	0.0007	124.2	25.9	4.1	2.2
Metazachlor	0.01	0.07	107.6	28.6	2.8	1.1
Methadone	0.0006	0.001	100.1	10.8	1.7	0.3
Methamphetamine	0.0002	0.0009	150.0	15.7	2.2	0.3
Methylparaben	0.01	0.05	106.9	12.1	5.0	2.0
Morphine	0.001	0.005	104.9	16.7	8.1	7.3
Nordiazepam	0.005	0.03	115.2	15.9	2.0	1.3
Norephedrine	0.003	0.02	75.5	1.6	2.4	0.4
Normorphine	0.001	0.002	39.8	9.9	12.1	10.1
Nortriptyline	0.001	0.006	91.8	6.7	2.4	1.1
O-Desmethylnaproxen	*0.7*	*2*	*109.0*	*2.0*	*5.1*	*4.0*
Omeprazole	0.005	0.01	121.9	15.4	1.1	0.6
Oxadiazon	0.007	0.01	731.2	362.3	2.3	2.7
Oxycodone	0.06	0.1	106.1	22.3	2.4	0.9
Oxymorphone	*0.0003*	*0.002*	*1143.7*	*597.5*	*4.8*	*6.1*
Pholcodine	n/a	n/a	78.4	18.9	6.5	2.3
Praziquantrel	0.0001	0.0001	125.1	16.7	4.1	1.1
Propylparaben	0.05	0.1	111.0	25.6	7.4	0.9
Quetiapine	0.00004	0.0002	114.2	29.0	2.4	0.9
Risperidone	0.0008	0.002	426.6	191.5	1.4	0.4
Salbutamol	0.007	0.01	122.5	33.0	2.2	0.2
Sotalol	0.1	0.4	649.6	345.1	1.9	1.2
Sulphadiazine	0.001	0.002	109.8	4.5	4.7	3.1
Sulphamethoxazole	0.0005	0.001	69.1	11.5	6.0	1.7
Sulphapyridine	0.001	0.007	87.9	12.7	1.9	0.5
Terbutaline	0.001	0.002	-15.4	15.3	-0.5	0.2
Terbuthylazine	0.01	0.05	584.4	173.4	2.7	1.4
Tetramisole	0.006	0.01	119.1	20.0	4.4	1.6
Thiamethoxam	*0.000004*	*0.00002*	*61.8*	*10.3*	*3.9*	*1.7*
Triclosan	*0.05*	*0.1*	*103.5*	*2.6*	*7.6*	*5.6*
Valsartan	0.06	0.1	87.0	9.9	14.9	8.6
Vardenafil	0.0007	0.001	510.1	286.9	0.8	0.3
Zolpidem	0.3	0.8	82.4	12.9	11.8	12.3

Table 4. Method limits of detection, quantification, method accuracy and method precision in influent wastewater (n=9) (semiquantitative compounds are presented in italics)

Analyte	Wastewater					
	mLOD ($\mu g\ L^{-1}$)	mLOQ ($\mu g\ L^{-1}$)	Average Accuracy		Average Precision	
			%	SD	%	SD
Aminorex	0.005	0.009	105.3	22.6	2.4	2.0
Anhydroecgonine methylester	0.0001	0.0003	111.6	11.1	2.0	1.6
Benzophenone-1	0.01	0.07	66.0	9.8	14.2	8.8
Benzophenone-4	0.2	0.5	107.6	9.0	5.4	2.1
Benzoylecgonine	0.0001	0.0005	101.2	18.6	1.1	0.4
Benzylpiperizine	*0.002*	*0.008*	*74.5*	*8.5*	*3.4*	*3.0*
Bezafibrate	0.01	0.07	99.9	8.9	11.5	6.3
Buprenorphine	0.006	0.03	10.0	0.7	8.0	5.2
Candesartan Cilexetil	*0.0001*	*0.0005*	*1398.0*	*610.8*	*4.8*	*1.6*
Carbamazepine	0.001	0.006	120.6	13.9	0.7	0.2
Carbamazepine 10,11 epoxide	0.001	0.005	104.0	22.3	2.1	1.0
Carprofen	*0.2*	*0.5*	*116.9*	*25.9*	*9.5*	*1.7*
Citalopram	0.001	0.006	203.4	63.2	2.5	0.4
Clothiniadin	0.0006	0.001	101.0	9.9	2.7	0.3
Cocaethylene	0.0006	0.001	120.4	14.1	1.7	0.8
Cocaine	0.0006	0.001	118.7	17.5	1.2	0.5
Codeine	0.0005	0.001	169.9	52.4	3.2	1.6
Cotinine	0.007	0.04	49.3	146.7	1.6	0.5
Desmethylcitalopram	0.01	0.06	907.1	554.9	1.6	0.0
DHMA	*0.1*	*0.2*	*117.6*	*17.3*	*3.9*	*3.0*
Diazepam	0.006	0.01	110.3	9.8	5.2	1.8
Diazinon	0.0007	0.001	336.4	80.7	5.2	2.4
Diclofenac	0.01	0.05	132.3	23.4	7.0	6.3
Dihydrocodeine	0.02	0.08	128.9	20.4	2.2	1.1
Dihydroketoprofen	0.2	0.5	135.7	46.8	7.5	2.8
Dihydromorphine	0.003	0.02	80.4	13.9	4.0	1.9
Diltiazem	*0.001*	*0.002*	*1007.7*	*634.0*	*1.6*	*0.5*
Duloxetine	*0.0006*	*0.003*	*387.4*	*98.1*	*5.4*	*1.8*
E1-10,11-dihydro-10-hydroxycarbamazepine	0.04	0.1	109.0	18.8	1.3	0.3
E1-Alprenolol	0.0008	0.004	107.8	12.7	2.4	1.1
E1-Atenolol	0.003	0.006	116.2	14.1	1.1	0.2
E1-Bisoprolol	0.0006	0.003	99.4	20.7	2.3	1.6
E1-Metoprolol	0.003	0.0006	85.9	11.8	1.3	0.3
E1-Mirtazapine	0.0001	0.006	220.5	120.8	4.2	2.8
E1-Oxazepam	0.0006	0.003	103.1	4.1	8.0	2.2
E1-Propanolol	0.005	0.01	90.7	13.3	2.0	1.2
E1-Tramadol	*0.007*	*0.01*	*165.5*	*64.8*	*4.6*	*0.7*
E2-10,11-dihydro-10-hydroxycarbamazepine	0.005	0.01	109.0	13.8	3.3	0.7
E2-Alprenolol	0.0006	0.003	100.7	9.5	3.9	2.4
E2-Atenolol	0.003	0.006	112.8	9.4	1.2	0.8
E2-Bisoprolol	0.003	0.005	99.8	20.3	1.3	0.8
E2-Metoprolol	0.01	0.06	116.1	13.6	4.5	4.5
E2-Mirtazapine	0.0001	0.007	164.2	36.2	4.6	4.2
E2-Oxazepam	0.001	0.002	104.0	11.7	5.8	3.0
E2-Propanolol	0.003	0.05	133.1	16.8	1.4	1.0
Ethylparaben	0.6	1	122.0	16.3	4.5	2.9
Fexofenadine	*0.3*	*0.6*	*133.2*	*39.8*	*9.1*	*1.1*
Griseofulvin	0.008	0.02	91.8	14.8	3.4	1.5
Heroin	0.001	0.006	125.5	10.5	4.8	0.8

HMA	0.008	0.02	83.6	4.4	3.0	1.3
HMMA	*0.0004*	*0.0008*	*69.9*	*7.5*	*2.3*	*2.5*
Hydrocodone	0.0008	0.002	105.7	15.7	2.1	0.8
Imatinib	0.004	0.008	1191.8	445.9	1.2	0.6
Imidacloprid	0.001	0.003	56.0	7.4	3.2	2.1
Indoprofen	0.002	0.008	99.1	15.6	3.9	3.4
Iopromide	*0.001*	*0.006*	*148.0*	*20.8*	*8.7*	*5.5*
Ketamine	0.001	0.006	104.7	12.0	2.1	0.8
Ketoprofen	0.4	0.9	110.7	2.9	4.0	3.2
MDA	0.001	0.006	113.4	34.6	1.5	0.2
MDMA	0.001	0.006	85.9	16.9	1.2	0.3
MDPV	0.0001	0.0007	116.1	13.3	2.0	0.6
Memantine	*0.006*	*0.01*	*82.9*	*17.4*	*4.4*	*0.5*
Mephedrone	0.0002	0.0009	148.7	21.7	0.8	0.5
Metazachlor	0.008	0.04	122.9	15.2	5.2	4.1
Methadone	0.0007	0.001	143.7	25.0	2.6	0.5
Methamphetamine	0.0001	0.0007	118.7	31.3	2.1	0.5
Methylparaben	0.01	0.05	103.5	9.2	2.6	0.3
Morphine	0.001	0.005	31.7	107.8	2.5	1.4
Nordiazepam	0.002	0.009	100.6	14.4	3.0	1.6
Norephedrine	0.002	0.009	90.2	2.8	2.6	0.5
Normorphine	0.0003	0.0006	42.1	14.7	2.1	0.3
Nortriptyline	0.001	0.006	82.0	10.2	5.3	1.6
O-Desmethylnaproxen	*0.2*	*0.6*	*98.7*	*26.0*	*6.4*	*3.0*
Omeprazole	0.01	0.03	89.2	14.5	2.1	0.7
Oxadiazon	0.007	0.01	633.6	308.4	6.2	0.7
Oxycodone	0.008	0.02	97.2	32.0	8.6	10.1
Oxymorphone	*0.0002*	*0.0009*	*303.9*	*93.7*	*5.3*	*1.2*
Pholcodine	0.006	0.03	50.7	13.3	10.0	7.1
Praziquantrel	0.0001	0.0001	110.7	15.3	5.6	1.0
Propylparaben	0.08	0.2	124.9	13.1	2.0	2.3
Quetiapine	0.00001	0.00006	96.5	36.8	2.3	0.3
Risperidone	0.0007	0.001	361.9	153.9	1.3	0.4
Salbutamol	0.007	0.01	96.7	20.3	1.0	0.5
Sotalol	0.3	0.7	421.5	114.2	2.5	1.6
Sulphadiazine	0.0007	0.001	88.7	6.9	3.5	0.8
Sulphamethoxazole	0.0005	0.0009	67.3	3.4	2.1	0.3
Sulphapyridine	0.0009	0.005	128.4	2.7	1.4	0.2
Terbutaline	0.001	0.002	-17.9	17.7	-1.0	0.7
Terbuthylazine	0.01	0.05	506.5	137.4	1.3	0.6
Tetramisole	0.007	0.01	121.8	11.8	7.1	6.5
Thiamethoxam	*0.000003*	*0.00001*	*47.1*	*8.8*	*6.7*	*4.1*
Triclosan	*0.08*	*0.2*	*170.8*	*125.2*	*2.8*	*3.1*
Valsartan	0.08	0.2	82.1	2.8	5.0	3.9
Vardenafil	0.0005	0.0009	425.1	166.4	1.8	0.4
Zolpidem	0.06	0.2	64.2	4.7	13.8	11.2

Semi-quantitative compounds again appear in italics. Likewise, precision results should be as close to zero as possible and should be < 20 % RSD. Most analytes performed well with accuracies between 80-120 % and precision of <20 % (Table 3 and 4). Exceptions include candesartan cilexetil, diltiazem, duloxetine, fexofenadine and thiamethoxam. The only analytes with method precision of >20 % were DHMA and dihydroketoprofen in river water only, with an average precision of 21%.

Resolution of chiral compounds and enantiomeric fractions were calculated at three concentrations and the average results are presented in Table 7. Resolution was excellent in both matrices, although generally better in river water; due to narrower peak widths and greater S/N from a "cleaner" matrix. 10,11-dihydro-10-hydroxycarbamazepine had better resolution in wastewater than river water as the E1-enantiomer did not always have a quantifiable signal to noise (S/N) ratio in the latter matrix. This, coupled with a narrow peak width, lead to relatively greater separation of the two enantiomers in wastewater, rather than in river water where both were detected with a quantifiable S/N and a broader peak width.

Table 5. Method resolution of enantiomers and enantiomeric fractions (n=9)

Analyte	River water				Wastewater			
	Rs	**SD**	**EF**	**SD**	**Rs**	**SD**	**EF**	**SD**
10,11-dihydro-10-hydroxycarbamazepine	13.42	2.34	0.45	0.07	28.56	22.11	0.41	0.09
Alprenolol	21.92	4.73	0.52	0.01	14.90	2.52	0.45	0.05
Atenolol	20.84	4.26	0.48	0.02	9.48	5.80	0.50	0.02
Bisoprolol	46.42	30.58	0.53	0.01	24.82	8.03	0.54	0.02
Metoprolol	50.22	44.11	0.52	0.02	14.58	2.81	0.50	0.01
Mirtazapine	7.07	2.02	0.51	0.01	6.43	3.17	0.52	0.01
Oxazepam	6.54	2.01	0.53	0.03	13.89	11.71	0.45	0.13
Propanolol	46.41	36.90	0.42	0.11	14.32	4.89	0.47	0.04
Tramadol	3.76	1.30	-	-	5.08	1.35	-	-

In summary, after validation, out of 140 analytes tested, there were eighty-one compounds where fully quantitative information could be determined, and fourteen semi-quantitatively analysed compounds: benzylpiperazine, candesartan cilexetil, carprofen, DHMA, diltiazem, duloxetine, E1-tramadol, fexofenadine, HMMA, iopromide, memantine, oxymorphone, thiamethoxam and triclosan. There was no clear difference between pKa and Log P of the fully and semi-quantiative analytes, although the semi-quantitative analytes had a slightly higher average log P (3.4 ± 2.5 vs 2.4 ± 1.6 respectively). Likewise, there was no significant difference between the Log P and pKa of the 45 qualitative analytes and the 95 quantitative or semi-quantitative analytes. Several of these qualitative analytes performed poorly with

very low or very high average relative recoveries, despite using a deuterated analogue of the analyte as the internal standard and good instrument performance results.

The ninety-five semi- or fully quantitative compounds included analytes from a range of environmentally important classes including seven herbicides, insecticides and pesticides, which enter the environment directly (without wastewater treatment) as run-off from agriculture, as well as five antifungal compounds, that are routinely added to personal care products. Additionally, carprofen and sulphapyridine are licensed for veterinary use and may also enter the environment directly. Most of the other analytes are primarily classified as human pharmaceuticals including, antidepressants, beta-blockers, non-steroidal anti-inflammatory drugs (NSAIDs) and opioids. In particular, the beta-blockers performed very well in this method, and were all fully quantitative, which was expected as the SFC method was selected because it effectively separated beta-blockers. Monitoring these pharmaceuticals is important for assessing both public health, via influent wastewater, and environmental health, via effluent wastewater and river water, particularly considering European directives concerning water quality and reducing the environmental impact of human pharmaceutical usage (European Parliament & Council, 2002, Commission, 2019). The remaining fourteen analytes are primarily classified as drugs of abuse or their metabolites, although ketamine is also widely used in veterinary medicine and so may also enter the environment directly. Whilst these compounds are primarily of interest for monitoring drug consumption within human populations, they are also analogous to other pharmaceuticals as potential compounds of environmental concern.

3.3 Environmental analysis

Environmental samples were analysed using the validated method. Average concentrations in each matrix were recorded in Table 6. The average enantiomeric fraction and average peak resolution for chiral analytes in each matrix were presented in Table 7.

Table 6. Analysis of environmental samples in river water, effluent and influent wastewater (semiquantitative compounds are presented in italics)

Analyte	River water		Effluent		Influent	
	Average concentration (ng L^{-1}) (n=6)	SD	Average concentration (ng L^{-1}) (n=6)	SD	Average concentration (ng L^{-1}) (n=6)	SD
Aminorex	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Anhydroecgonine methylester	<LOQ	<LOQ	2873.3	42.5	2371.7	25.1
Benzophenone-1	<LOQ	<LOQ	<LOQ	<LOQ	1211.7	24.0
Benzophenone-4	2101.7	20.7	60825.0	6.5	18363.3	10.9
Benzoylecgonine	538.3	4.1	7998.3	1.4	22773.3	2.2
Benzylpiperizine	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	*217.5*	*5.4*
Bezafibrate	<LOQ	<LOQ	9781.7	42.7	8821.7	21.3
Buprenorphine	290.8	14.3	295.0	18.8	307.5	17.4
Candesartan Cilexetil	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*
Carbamazepine	311.7	6.3	5325	1.6	4191.7	1.4
Carbamazepine 10,11 epoxide	<LOQ	<LOQ	<LOQ	<LOQ	237.5	34.9
Carprofen	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Citalopram	826.7	3.6	6176.7	3.6	5083.3	1.8
Clothiniadin	486.7	5.1	168.3	16.2	150.0	14.4
Cocaethylene	<LOQ	<LOQ	<LOQ	<LOQ	285.0	7.8
Cocaine	<LOQ	<LOQ	1698.3	2.5	9338.3	2.7
Codeine	988.3	6.7	24883.3	3.7	20900.0	3.2
Cotinine	260.0	4.4	6206.67	0.5	15716.7	2.3
Desmethyl citalopram	335.0	6.6	1693.3	5.9	1381.7	5.2
DHMA	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*
Diazepam	<LOQ	<LOQ	50.0	54.2	20.0	70.7
Diazinon	242.5	29.7	<LOQ	<LOQ	161.7	12.6
Diclofenac	<LOQ	<LOQ	8988.3	40.4	1648.4	20.9
Dihydrocodeine	236.7	7.2	4298.3	11.2	2951.7	4.8
Dihydro ketoprofen	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Dihydromorphine	<LOQ	<LOQ	511.7	4.8	538.3	5.8
Diltiazem	*266.7*	*1.8*	*1033.3*	*3.9*	*1083.3*	*3.7*
Duloxetine	*130.0*	*6.3*	*236.7*	*9.3*	*253.3*	*15.6*
E1-10,11-dihydro-10-hydroxy-carbamazepine	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
E1-Alprenolol	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
E1-Atenolol	103.33	40.5	4468.33	2.4	6211.7	5.2
E1-Bisoprolol	<LOQ	<LOQ	791.67	9.4	808.33	5.3
E1-Metoprolol	<LOQ	<LOQ	61.7	49	65.0	34.1
E1-Mirtazapine	71.67	9.6	1003.3	3.8	621.7	5.4
E1-Oxazepam	118.3	37	856.7	18.1	396.7	18.6
E1-Propanolol	<LOQ	<LOQ	1170.0	6.2	916.7	9.4
E1-Tramadol	*401.7*	*6.8*	*3691.7*	*9*	*2348.3*	*3.1*
E2-10,11-dihydro-10-	<LOQ	<LOQ	2086.7	11.8	1031.7	8.8

hydroxy-carbamazepine						
E2-Alprenolol	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
E2-Atenolol	193.3	12.2	4583.3	5.4	5925	4.5
E2-Bisoprolol	113.3	4.2	1086.7	2.2	1003.3	4.6
E2-Metoprolol	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
E2-Mirtazapine	96.7	7.7	446.7	5.4	295.0	6.4
E2-Oxazepam	241.7	12	875.0	18.7	555	18
E2-Propanolol	245.0	8.7	1970	5.8	1436.7	4.5
Ethylparaben	511.7	2.6	548.3	6.3	2376.7	9.1
Fexofenadine	*<LOQ*	*<LOQ*	*27843.3*	*50.7*	*10281.7*	*21.8*
Griseofulvin	150.0	19.6	157.5	13.7	205	22.6
Heroin	305.0	5.3	<LOQ	<LOQ	343.33	15.4
HMA	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
HMMA	<LOQ	<LOQ	346.7	16.4	16.7	44.9
Hydrocodone	791.7	4.7	7540.0	2.3	5551.7	1.8
Imatinib	183.3	10.3	301.7	14.8	368.3	12.1
Imidacloprid	446.7	6.2	3091.7	5.8	788.3	7.3
Indoprofen	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Iopromide	*<LOQ*	*<LOQ*	*14861.7*	*35.3*	*<LOQ*	*<LOQ*
Ketamine	148.3	7.2	3026.7	5.5	2371.7	3.8
Ketoprofen	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
MDA	216.7	5.8	870.0	4.3	476.7	3.8
MDMA	31.7	11.8	2458.3	1.8	3945.0	2.2
MDPV	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Memantine	*<LOQ*	*<LOQ*	*391.7*	*27.7*	*226.7*	*9.8*
Mephedrone	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Metazachlor	299.7	3.9	278.3	5.3	276.7	1.7
Methadone	10.0	0.0	431.7	3.4	513.3	2.7
Methamphetamine	141.7	2.6	263.3	3.6	268.3	5.5
Methylparaben	371.7	27.9	440	23.2	16301.7	12
Morphine	328.3	5.4	8661.7	6.3	11210.0	2.7
Nordiazepam	55.0	9.1	230.0	14.6	171.7	14
Norephedrine	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Normorphine	731.67	2.1	1438.3	18.4	1810.0	9.6
Nortriptyline	563.3	10.9	923.3	5.1	616.7	7.8
O-Desmethy lnaproxen	<LOQ	<LOQ	25875.0	47.6	35981.7	21.3
Omeprazole	321.7	2.1	508.3	4.5	1468.3	3.3
Oxadiazon	536.7	30.6	<LOQ	<LOQ	<LOQ	<LOQ
Oxycodone	<LOQ	<LOQ	373.33	20.5	700	0.0
Oxymorphone	*<LOQ*	*<LOQ*	*455.0*	*19.5*	*435.0*	*13.6*
Pholcodine	<LOQ	<LOQ	44582.5	36.8	26923.3	44.2
Praziquantrel	15.0	50.9	90.0	9.1	28.3	51.6
Propylparaben	550.0	1.8	611.7	7.3	3583.3	7.6
Quetiapine	346.7	1.4	506.7	3.2	751.7	1.8
Risperidone	3683.3	16.5	4286.7	16.8	2440	13.8
Salbutamol	238.3	98.0	413.3	60.7	8078.0	215.4
Sotalol	6198.3	32.6	97220	9.3	44135.0	10.5
Sulphadiazine	<LOQ	<LOQ	288.3	38.7	<LOQ	<LOQ
Sulphamethox-azole	<LOQ	<LOQ	6426.7	9.0	2590.0	11.5
Sulphapyridine	496.7	5.5	18958.3	13.7	13751.7	4.5
Terbutaline	106647.0	17.9	136080.0	14.4	206545.0	12.7
Terbuthylazine	126.7	5.9	<LOQ	<LOQ	83.3	6.0
Tetramisole	<LOQ	<LOQ	233.3	9.5	256.7	15.0
Thiamethoxam	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*
Triclosan	*1325.0*	*9.4*	*1890.0*	*44.4*	*6185.0*	*31.0*
Valsartan	<LOQ	<LOQ	5481.7	47.0	2325.0	20.9

Vardenafil	2618.3	27.5	1691.7	33.5	1201.7	46.3
Zolpidem	190.0	0.4	240.0	10.6	<LOQ	<LOQ

Table 7. Average enantiomeric fraction and separation of chiral analytes in matrix ± standard deviation (n=9).

Analytes	River water		Effluent		Influent	
	EF	R_s	EF	R_s	EF	R_s
10,11-dihydro-10-hydroxycarbamazepine	<LOQ	18.2 ± 1.4	0.00 ± 0.01	14.6 ± 1.1	0.0 ± 0.01	14.5 ± 0.9
Alprenolol	<LOQ	5.4 ± 1.1	<LOQ	5.5 ± 0.4	<LOQ	5.3 ± 0.6
Atenolol	0.30 ± 0.10	30.5 ± 2.4	0.5 ± 0.01	30.3 ± 1.2	0.5 ± 0.01	32.4 ± 1.3
Bisoprolol	0.00 ± 0.01	10.1 ± 1.8	0.4 ± 0.01	7.9 ± 0.4	0.4 ± 0.01	7.6 ± 0.4
Metoprolol	<LOQ	<LOQ	1.0 ± 0.01	12.2 ± 4.7	1.0 ± 0.01	10.5 ± 0.9
Mirtazapine	0.40 ± 0.01	8.2 ± 0.8	0.7 ± 0.01	6.7 ± 0.2	0.7 ± 0.01	8.6 ± 0.7
Oxazepam	0.30 ± 0.10	33.2 ± 5.0	0.5 ± 0.10	19.7 ± 2.1	0.4 ± 0.10	25.1 ± 2.7
Propanolol	<LOQ	22.4 ± 2.8	0.4 ± 0.01	23.2 ± 1.3	0.4 ± 0.01	21.8 ± 1.2
Tramadol	-	6.0 ± 0.3	-	6.0 ± 0.1	-	5.9 ± 0.2

CECs were quantified in river water at concentrations spanning from <LOQ (aminorex, AEME, benzophenone-1, benzylpiperizine, candesartan cilexetil, carbamazepine-10,11-epoxide, carprofen, cocaethylene, cocaine, DHMA, diazepam, diclofenac, dihydroketoprofen, dihydromorphine, E1 & E2-10,11-hydrodyo-10-hydroxycarbamazepine, E1 & E2-alprenolol, E1 & E2-metoprolol, E1-propanolol, fexodenadine, HMA, HMMA, indoprofen, iopromide, ketoprofen, MDPV, memantine, mephedrone, norephedrine, O-desmethylnaproxen, oxycodone, oxymorphone, pholcodine, sulphadiazine, sulphamethoxazole, tetramisole, thiamethoxam and valsartan) through 0-988 ng L^{-1} (benzoylecgonine, buprenorphine, carbamazepine, citalopram, clothiniadin, codeine, cotinine, desmethylcitalopram, diazinon, dihydrocodeine, diltiazem, duloxetine, E1 & E2-atenolol, E1 & E2-mirtazapine, E1 & E2-oxazepam, E1-tramadol, E2-bisoprolol, E2-propanolol, ethylparaben, griseofulvin, heroin, hydrocodone, imatinib, imidacloprid, ketamine, MDA, MDMA, metazachlor, methadone, methamphetamine, methylparaben, morphine, nordiazepam, normorphine, nortriptyline, omeprazole, oxadiazon, praziquantrel, propylparaben, quetiapine, salbutamol, sulphapyridine, terbuthylazine and zolpidem) to 1-106 µg L^{-1} (benzophenone-4, risperidone, sotalol, terbutaline, triclosan and vardenafil). Interestingly, detected concentrations of some CECs were lower in wastewater influent (20 – 26,923 µg L^{-1}, average concentration 7402 µg L^{-1}) than in effluent wastewater (50 – 136,080 µg L^{-1}, average concentration 8260 µg L^{-1}), which may reflect influence from microbial metabolic processes during wastewater treatment. For example, a metabolite of citalopram (desmethylcitalopram) was found at concentrations of 1382 µg L^{-1} in wastewater influent and at 1693 µg L^{-1} in wastewater effluent. Likewise, oxazepam had greater concentrations in effluent wastewater (1732 µg L^{-1}) than in influent

wastewater (952 µg L^{-1}) which could results from it being a common metabolite of several other benzodiazepines, such as diazepam. However, as the water used in this experiment was collected by grab sampling it was not possible to conclusively say that this observation was due to metabolic processes occurring during wastewater treatment. Similarly, some analytes, such as oxadiazon and terbuthylazine, had a greater concentration in river water than influent or effluent wastewater. This is likely because they are used as pesticides and so are entering the environment directly, e.g. through run off from fields and gardens, rather than from human consumption.

Chiral CECs that were enantiomerically separated are presented in Table 7. Most analytes showed non racemic EFs, which indicates enantiomer selective processes occurring either due to human metabolism or microbial processes. This in turn highlights the importance of understanding chirality for determining biological effects, including toxicity. For example, bisoprolol was the only beta-blocker quantified in all three matrices and was also enriched with the E2 isomer in river water compared with influent and effluent wastewater. The EF of mirtazapine appeared to vary considerably between wastewater influent and effluent, and river water, which suggested it was being preferentially metabolised favouring the E1 enantiomer in humans and the E2 enantiomer in the environment. To the authors knowledge, there is no literature data on the ecotoxicity of mirtazapine, although other antidepressants have been studied [46; 47]. The EF of oxazepam also varied between the matrices, however the difference in EF was much less pronounced.

4. Conclusion

The development of new analytical methods for the analysis of environmental micropollutants is important, particularly where critical information on chirality can be collected. SFC is an excellent technique for combined achiral - chiral analysis as it allows for the development of robust methods with shorter run times than would usually be achieved in chiral HPLC methods. This is due to the combined use of supercritical CO_2, non-biological chiral selectors and smaller UHPLC size particles. The method development data shown highlighted the range of available SFC column chemistries, and optimised chromatographic conditions for the development of new, combined non-chiral and chiral-SFC methods capable of separating a range of different chiral and non-chiral analytes. The final method showcases the power of SFC for the rapid analysis (within <10min) of chiral and achiral compounds in important environmental matrices. Whilst this method was only able to chirally separate nine analytes the initial method development showed that under different chromatographic conditions the same column could partially or fully separate another five analytes, with others separated under the same chromatographic conditions using alternative columns. In summary, out of 140 analytes selected for the study, 81 were fully quantifiable and validated, and 14 were semi-quantitative. mLOQs spanned 10 pg L^{-1} – 2 µg L^{-1} and accuracy and precision were maintained at 103 ± 11.1 % and 4 ± 2.1 % respectively. The analysis of environmental samples showed omnipresence of selected CECs, some showing enantioselective fate, such as mirtazapine. Overall the CEL-1 methods gave excellent separation of chiral enantiomers and rapid quantitative analysis of 95 CEC, at the cost of reduced instrument and method sensitivity compared to contemporary achiral methodologies. However, these achiral methodologies also provide a road map for how to improve sensitivity without sacrificing the efficiency of SFC or focusing on only a handful of analytes.

Acknowledgments

The support of the Leverhulme Trust (Project No RPG-2013-297) and Engineering and Physical Sciences Research Council (Project No EP/P028403/1) is greatly appreciated. The authors would also like to thank Waters for support and provision of ACQUITY UPC2 System for the study.

References

[1] European Parliament & Council, 2000. Water Framwork Directive 2000/60/EC. *Official Journal of the European Union,* (327), pp. 1-73.

[2] Loos, R., 2012. Analytical methods relevant to the European Commission's 2012 proposal on Priority Substances under the Water Framework Directive. JRC scientific and policy reports: Luxembourg: Publications office of the European Union, 2012.

[3] European Commission, 2019. *European Union Strategic Approach to Pharmaceuticals in the Environment.*

[4] Institute for health and consumer protection, 2003. *Technical guidance document on risk assessment in support of Commission Directive 93/67/ EEC on risk assessment for new notified substances and Commission Regulation (EC) No. 1488/94 on risk assessment for existing substances.*: European Chemicals Bureau.

[5] Committee for Medicinal Products for Human Use, 2018. *Guideline on the environmental risk assessment of medicinal products for human use.*

[6] Bagnall, J.P., Evans, S.E., Wort, M.T., Lubben, A.T. and Kasprzyk-Hordern, B., 2012. Using chiral liquid chromatography quadrupole time-of-flight mass spectrometry for the analysis of pharmaceuticals and illicit drugs in surface and wastewater at the enantiomeric level. *Journal of Chromatography A,* 1249, pp. 115-129.

[7] Chiaia, A.C., Banta-Green, C. and Field, J., 2008. Eliminating Solid Phase Extraction with Large-Volume Injection LC/MS/MS: Analysis of Illicit and Legal Drugs and Human Urine Indicators in US Wastewaters. *Environmental Science & Technology,* 42(23), pp. 8841-8848.

[8] Diaz-Cruz, M.S. and Barcelo, D., 2006. Determination of antimicrobial residues and metabolites in the aquatic environment by liquid chromatography tandem mass spectrometry. *Analytical and Bioanalytical Chemistry,* 386(4), pp. 973-985.

[9] Fent, K., Weston, A.A. and Caminada, D., 2006. Ecotoxicology of human pharmaceuticals. *Aquatic Toxicology,* 76(2), pp. 122-159.

[10] Gross, B., Montgomery-Brown, J., Naumann, A. and Reinhard, M., 2004. Occurrence and fate of pharmaceuticals and alkylphenol ethoxylate metabolites in an effluent-dominated river and wetland. *Environmental Toxicology and Chemistry,* 23(9), pp. 2074-2083.

[11] Wang, C.C., Hou, L.L., Li, J., Xu, Z.Q., Gao, T.T., Yang, J., Zhang, H.F., Li, X.Q. and Du, P., 2017. Occurrence of diazepam and its metabolites in wastewater and surface waters in Beijing. *Environmental Science and Pollution Research,* 24(18), pp. 15379-15389.

[12] Winkler, M., Lawrence, J.R. and Neu, T.R., 2001. Selective degradation of ibuprofen and clofibric acid in two model river biofilm systems. *Water Research,* 35(13), pp. 3197-3205.

[13] Zuccato, E., Chiabrando, C., Castiglioni, S., Calamari, D., Bagnati, R., Schiarea, S. and Fanelli, R., 2005. Cocaine in surface waters: a new evidence-based tool to monitor community drug abuse. *Environmental health : a global access science source,* 4, pp. 14-14.

[14] Camacho-Munoz, D., Kasprzyk-Hordern, B. and Thomas, K.V., 2016. Enantioselective simultaneous analysis of selected pharmaceuticals in environmental samples by ultrahigh performance supercritical fluid based chromatography tandem mass spectrometry. *Analytica Chimica Acta,* 934, pp. 239-251.

[15] Kasprzyk-Hordern, B. and Baker, D.R., 2012. Enantiomeric Profiling of Chiral Drugs in Wastewater and Receiving Waters. *Environmental Science & Technology,* 46(3), pp. 1681-1691.

[16] Petrie, B., Rood, S., Smith, B.D., Proctor, K., Youdan, J., Barden, R. and Kasprzyk-Hordern, B., 2018. Biotic phase micropollutant distribution in horizontal sub-surface flow constructed wetlands. *Science of the Total Environment,* 630, pp. 648-657.

[17] Petrie, B., Youdan, J., Barden, R. and Kasprzyk-Hordern, B., 2016. Multi-residue analysis of 90 emerging contaminants in liquid and solid environmental matrices by ultra-high-performance liquid chromatography tandem mass spectrometry. *Journal of Chromatography A,* 1431, pp. 64-78.

[18] Petrovic, M., Hernando, M.D., Diaz-Cruz, M.S. and Barcelo, D., 2005. Liquid chromatography-tandem mass spectrometry for the analysis of pharmaceutical residues in environmental samples: a review. *Journal of Chromatography A,* 1067(1-2), pp. 1-14.

[19] Corcoran, J., Winter, M.J. and Tyler, C.R., 2010. Pharmaceuticals in the aquatic environment: A critical review of the evidence for health effects in fish. *Critical Reviews in Toxicology,* 40(4), pp. 287-304.

[20] Filby, A.L., Paull, G.C., Searle, F., Ortiz-Zarragoitia, M. and Tyler, C.R., 2012. Environmental Estrogen-Induced Alterations of Male Aggression and Dominance Hierarchies in Fish: A Mechanistic Analysis. *Environmental Science & Technology,* 46(6), pp. 3472-3479.

[21] Memmert, U., Peither, A., Burri, R., Weber, K., Schmidt, T., Sumpter, J.P. and Hartmann, A., 2013. DICLOFENAC: NEW DATA ON CHRONIC TOXICITY AND BIOCONCENTRATION IN FISH. *Environmental Toxicology and Chemistry,* 32(2), pp. 442-452.

[22] Rat'ko, A.A., Stefan, R.L., van Staden, J.F. and Aboul-Enein, H.Y., 2004. Macrocyclic antibiotics as chiral selectors in the design of enantio selective, potentiometric membrane electrodes for the determination of L- and D-enantiomers of methotrexate. *Talanta,* 64(1), pp. 145-150.

[23] Hofstetter, R.K., Potlitz, F., Schulig, L., Kim, S., Hasan, M. and Link, A., 2019. Subcritical Fluid Chromatography at Sub-Ambient Temperatures for the Chiral Resolution of Ketamine Metabolites with Rapid-Onset Antidepressant Effects. *Molecules,* 24(10).

[24] Hoguet, V., Charton, J., Hecquet, P.-E., Lakhmi, C. and Lipka, E., 2018. Supercritical fluid chromatography versus high performance liquid chromatography for enantiomeric and diastereoisomeric separations on coated polysaccharides-based stationary phases: Application to dihydropyridone derivatives. *Journal of Chromatography A,* 1549, pp. 39-50.

[25] Hofstetter, R.K., Hasan, M., Eckert, C. and Link, A., 2019. Supercritical fluid chromatography: From science fiction to scientific fact. *Chemtexts,* 5(3).

[26] Waters, 2012. Simultaneous Enantiomeric Separation of Multiple Beta Blockers Using UltraPerformance Convergence Chromatography (UPC2) Waters Corporation.

[27] Ort, C., van Nuijs, A.L.N., Berset, J.-D., Bijlsma, L., Castiglioni, S., Covaci, A., de Voogt, P., Emke, E., Fatta-Kassinos, D., Griffiths, P., Hernandez, F., Gonzalez-Marino, I., Grabic, R., Kasprzyk-Hordern, B., Mastroianni, N., Meierjohann, A., Nefau, T., Oestman, M., Pico, Y., Racamonde, I., Reid, M., Slobodnik, J., Terzic, S., Thomaidis, N. and Thomas, K.V., 2014. Spatial differences and temporal changes in illicit drug use in Europe quantified by wastewater analysis. *Addiction,* 109(8), pp. 1338-1352.

[28] Gao, J.F., O'Brien, J., Du, P., Li, X.Q., Ort, C., Mueller, J.F. and Thai, P.K., 2016. Measuring selected PPCPs in wastewater to estimate the population in different cities in China. *Science of the Total Environment,* 568, pp. 164-170.

[29] Lai, F.Y., O'Brien, J., Bruno, R., Hall, W., Prichard, J., Kirkbride, P., Gartner, C., Thai, P., Carter, S., Lloyd, B., Burns, L. and Mueller, J., 2016. Spatial variations in the consumption of illicit stimulant drugs across Australia: A nationwide application of wastewater-based epidemiology. *Science of the Total Environment,* 568, pp. 810-818.

[30] Causanilles, A., Ruepert, C., Ibanez, M., Emke, E., Hernandez, F. and de Voogt, P., 2017. Occurrence and fate of illicit drugs and pharmaceuticals in wastewater from two wastewater treatment plants in Costa Rica. *Science of the Total Environment,* 599, pp. 98-107.

[31] Subedi, B. and Kannan, K., 2014. Mass Loading and Removal of Select Illicit Drugs in Two Wastewater Treatment Plants in New York State and Estimation of Illicit Drug Usage in Communities through Wastewater Analysis. *Environmental Science & Technology,* 48(12), pp. 6661-6670.

[32] Lopardo, L., Petrie, B., Proctor, K., Youdan, J., Barden, R. and Kasprzyk-Hordern, B., 2019. Estimation of community-wide exposure to bisphenol A via water fingerprinting. *Environment International,* 125, pp. 1-8.

[33] Phung, D., Mueller, J., Lai, F.Y., O'Brien, J., Dang, N., Morawska, L. and Thai, P.K., 2017. Can wastewater-based epidemiology be used to evaluate the health impact of temperature? - An exploratory study in an Australian population. *Environmental Research,* 156, pp. 113-119.

[34] Yuan, S.F., Liu, Z.H., Huang, R.P., Yin, H. and Dang, Z., 2016. Levels of six antibiotics used in China estimated by means of wastewater-based epidemiology. *Water Science and Technology,* 73(4), pp. 769-775.

[35] Sims, N., Rice, J. and Kasprzyk-Hordern, B., 2019. An ultra-high-performance liquid chromatography tandem mass spectrometry method for oxidative stress biomarker analysis in wastewater. *Analytical and Bioanalytical Chemistry,* 411(11), pp. 2261-2271.

[36] Ryu, Y., Gracia-Lor, E., Bade, R., Baz-Lomba, J.A., Bramness, J.G., Castiglioni, S., Castrignano, E., Causanilles, A., Covaci, A., de Voogt, P., Hernandez, F., Kasprzyk-Hordern, B., Kinyua, J., McCall, A.K., Ort, C., Plosz, B.G., Ramin, P., Rousis, N.I., Reid, M.J. and Thomas, K.V., 2016. Increased levels of the oxidative stress biomarker 8-iso-prostaglandin F-2 alpha in wastewater associated with tobacco use. *Scientific Reports,* 6.

[37] Depaolini, A.R., Fattore, E., Cappelli, F., Pellegrino, R., Castiglioni, S., Zuccato, E., Fanelli, R. and Davoli, E., 2016. Source discrimination of drug residues in wastewater: The case of salbutamol. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences,* 1023, pp. 62-67.

[38] Gonzalez-Marino, I., Gracia-Lor, E., Rousis, N.I., Castrignano, E., Thomas, K.V., Quintana, J.B., Kasprzyk-Hordern, B., Zuccato, E. and Castiglioni, S., 2016. Wastewater-Based Epidemiology To Monitor Synthetic Cathinones Use in Different European Countries. *Environmental Science & Technology,* 50(18), pp. 10089-10096.

[39] Thomas, K.V., Bijlsma, L., Castiglioni, S., Covaci, A., Emke, E., Grabic, R., Hernandez, F., Karolak, S., Kasprzyk-Hordern, B., Lindberg, R.H., de Alda, M.L., Meierjohann, A., Ort, C., Pico, Y., Quintana, J.B., Reid, M., Rieckermann, J., Terzic, S., van Nuijs, A.L.N. and de Voogt, P., 2012. Comparing illicit drug use in 19 European cities through sewage analysis. *Science of the Total Environment,* 432, pp. 432-439.

[40] van Wel, J.H.P., Gracia-Lor, E., van Nuijs, A.L.N., Kinyua, J., Salvatore, S., Castiglioni, S., Bramness, J.G., Covaci, A. and Van Hal, G., 2016. Investigation of agreement between wastewater-based epidemiology and survey data on alcohol and nicotine use in a community. *Drug and Alcohol Dependence,* 162, pp. 170-175.

[41] Swann, T., Berthelette, K., Fairchild, J. and Hill, J., 2015. Using blends of solvents and additives to enhance SFC chiral method development screening. Waters Corporation, 31/03/2020].

[42] European medicines agency, 1995. *ICH Topic Q 2 (R1), Validation of Analytical Procedures: Text and Methodology.*

[43] European Commission, 2002. *Implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results.* Official Journal of the European Communities.

[44] Castrignano, E., Lubben, A. and Kasprzyk-Hordern, B., 2016. Enantiomeric profiling of chiral drug biomarkers in wastewater with the usage of chiral liquid chromatography coupled with tandem mass spectrometry. *Journal of Chromatography A,* 1438, pp. 84-99.

[45] Evans, S.E., Davies, P., Lubben, A., and Kasprzyk-Hordern, B., 2015. Determination of chiral pharmaceuticals and illicit drugs in wastewater and sludge using microwave assisted extraction, solid-phase extraction and chiral liquid chromatography coupled with tandem mass spectrometry. *Analytica Chimica Acta,* 882, pp. 112-126.

[46] Silva, L.J.G., Pereira, A.M.P.T., Meisel, L.M., Lino, C.M. and Pena, A., 2015. Reviewing the serotonin reuptake inhibitors (SSRIs) footprint in the aquatic biota: Uptake, bioaccumulation and ecotoxicology. *Environmental Pollution,* 197, pp. 127-143.

[47] Fong, P.P. and Ford, A.T., 2014. The biological effects of antidepressants on the molluscs and crustaceans: A review. *Aquatic Toxicology,* 151, pp. 4-13.

Supplementary material
A multi-residue method by supercritical fluid chromatography coupled with tandem mass spectrometry method for the analysis of chiral and non-chiral chemicals of emerging concern in environmental samples

Jack Rice[a], *Anneke Lubben*[b], *Barbara Kasprzyk-Hordern*[a*]

[a] Department of Chemistry, Faculty of Science, University of Bath, Bath, BA2 7AY, United Kingdom (UK)
[b] Material and Chemical Characterisation Facility, University of Bath, Bath, BA2 7AY, United Kingdom (UK)
***** Corresponding author: Barbara Kasprzyk-Hordern, E-mail: b.kasprzyk-hordern@bath.ac.uk

Table S1. Supplier information and CAS numbers for all analytes and internal standards used in this paper

Compound	CAS number	Supplier
1,7-dimethylxantine	611-59-6	Sigma Aldrich
1S,2R-(+)-ephedrine D3	285979-73-9	LGC standards (Middlesex, UK)
2-Hydroxyibuprofen	51146-55-5	Sigma Aldrich (Gillingham, UK)
2-Phenylproponic acid	492-37-5	Sigma-Aldrich (Gillingham, UK)
8-isoF2B	177020-26-7	Cayman Chemicals
Acetaminophen	103-90-2	Sigma Aldrich
Acetaminophen D4	64315-36-2	Sigma Aldrich (Gillingham, UK)
AEME	43021-26-7	Sigma Aldrich (Cerilliant product)
Aminorex	2207-50-3	Sigma Aldrich (Gillingham, UK)
Amoxicillin	26787-78-0	Sigma Aldrich
Amphetamine	300-62-9	LGC (Cerilliant product)
Amphetamine D5	136765-27-0	LGC standards (Middlesex, UK)
Ampicillin	69-53-4	Sigma Aldrich
Azathioprine	446-86-6	Sigma Aldrich
Azithromycin	83905-01-5	Sigma Aldrich
Benzophenone-1	131-56-6	Sigma Aldrich
Benzophenone-2	131-55-5	Sigma Aldrich
Benzophenone-3	131-57-7	Sigma Aldrich
Benzophenone-4	4065-45-6	Sigma Aldrich
Benzoylecgonine	519-09-5	Sigma Aldrich
Benzoylecgonine D8	205446-21-5	LGC standards (Middlesex, UK)
Benzylpiperizine	2759-28-6	LGC
Bezafibrate	41859-67-0	Sigma Aldrich
Bicalutamide	90357-06-5	Sigma Aldrich
Buprenorphine	52485-79-7	Sigma Aldrich
Caffeine	58-08-2	Sigma Aldrich
Candesartan cilexetil	145040-37-5	Sigma Aldrich
Capecitabine	154361-50-9	Sigma Aldrich
Carbamazepine	298-46-4	Sigma Aldrich
Carbamazepine-10,11-epoxide	36507-30-9	LGC
Carbamazepine 13C6	298-46-4	Sigma Aldrich (Gillingham, UK)
Carboxyibuprofen	15935-54-3	Sigma Aldrich (Gillingham, UK)
Carprofen	53716-49-7	Sigma Aldrich (Gillingham, UK)
Cetirizine	83881-51-0	LGC
Chloramphenicol	56-75-7	Sigma Aldrich
Chlorpyrifos	2921-88-2	LGC (Dr Ehrenstorfer)
Cimetidine	51481-61-9	Sigma Aldrich
Citalopram	59729-33-8	Sigma Aldrich (Gillingham, UK)
Citalopram D6	1190003-26-9	Toronto Research Chemicals Inc. (Ontario, Canada)
Clarithromycin	81103-11-9	Sigma Aldrich
Clothiniadin	210880-92-5	Sigma Aldrich (PESTANAL)
Cocaethylene	529-38-4	Sigma Aldrich (Cerilliant product)
Cocaethylene D3	136765-30-5	LGC standards (Middlesex, UK)
Cocaine	50-36-2	LGC (Cerilliant product)
Cocaine D3	65266-73-1	LGC standards (Middlesex, UK)
Codeine	76-57-3	Sigma Aldrich
Codeine D6	1007844-34-9	LGC standards (Middlesex, UK)
Cotinine	486-56-6	Sigma Aldrich (Cerilliant product)
Cotinine D3	110952-70-0	LGC standards (Middlesex, UK)
Creatinine	60-27-5	Sigma Aldrich
Cytarabine	147-94-4	Sigma Aldrich
Danofloxacin	112398-08-0	Sigma Aldrich
Desmethylcitalopram	62498-67-3	Toronto Research Chemicals Inc. (Ontario, Canada)
Desmethylvenlafaxine	93413-62-8	Sigma Aldrich (Gillingham, UK)
Desvenlafaxine	300827-87-6	Sigma Aldrich
DHMA	15398-87-5	Kinesis
Diazepam	439-14-5	Sigma Aldrich (Cerilliant product)

Diazepam D5	65854-76-4	Toronto Research Chemicals Inc. (Ontario, Canada)
Diazinon	333-41-5	Sigma Aldrich (PESTANAL)
Dichlofluanid	1085-98-9	Sigma Aldrich (PESTANAL)
Diclofenac	15307-86-5	Sigma Aldrich
Dihydrocodeine	125-28-0	Sigma Aldrich
Dihydro ketoprofen	55453-87-7	Toronto Research Chemicals Inc. (Ontario, Canada)
Dihydromorphine	509-60-4	Sigma Aldrich (Cerilliant product)
Diltiazem	42399-41-7	Sigma Aldrich
Donepezil	120014-06-4	LGC
Duloxetine	116539-59-4	LGC
E1 & E2-10,11-dihydro-10-hydroxycarbamazepine	29331-92-8	LGC
E1 & E2-Alprenolol	13707-88-5	Sigma Aldrich (Gillingham, UK)
E1 & E2-Atenolol	29122-68-7	Sigma Aldrich (Gillingham, UK)
E1 & E2-Atenolol D7	1202864-50-3	Sigma Aldrich (Gillingham, UK)
E1 & E2-Bisoprolol	66722-44-9	Sigma Aldrich
E1 & E2-Metoprolol	51384-51-1	Sigma Aldrich (Gillingham, UK)
E1 & E2-Mirtazapine	85650-52-8	Sigma Aldrich
E1 & E2-Mirtazapine D3	1216678-68-0	Toronto Research Chemicals Inc. (Ontario, Canada)
E1 & E2-Propanolol	525-66-6	Sigma Aldrich
E1 & E2-Tramadol	27203-92-5	Sigma Aldrich (Gillingham, UK)
E1& E2-Metoprolol D7	12929006-91-2	Toronto Research Chemicals Inc. (Ontario, Canada)
E1& E2-Oxazepam	604-75-1	Sigma Aldrich (Cerilliant product)
E1& E2-Oxazepam D5	65854-78-6	Sigma Aldrich (Cerilliant product)
E1& E2-Propanolol D7	344298-99-3	Sigma Aldrich (Gillingham, UK)
Ecgonine methyl ester D3	136765-34-9	Sigma Aldrich (Cerilliant product)
EE2	57-63-6	Sigma Aldrich
Ephedrine	50-98-6	Sigma Aldrich
Erythromycin	114-07-8	Sigma Aldrich
Ethylparaben	120-47-8	Sigma Aldrich
Fexofenadine	83799-24-0	LGC
Flufenacet	142459-58-3	LGC (Dr Ehrenstorfer)
Flumequine	42835-25-6	Sigma Aldrich
Fluoxetine	54910-89-3	LGC (Cerilliant product)
Fluoxetine D5	1173020-43-3	Toronto Research Chemicals Inc. (Ontario, Canada)
Furosemide	54-31-9	Sigma Aldrich
Gabapentin	60142-96-3	LGC (Cerilliant product)
Gabapentin D4	1185039-20-6	TRC (Toronto Research Chemicals, Toronto, Canada)
Gemfibrozil	25812-30-0	Sigma Aldrich
Gliclazide	21187-98-4	LGC
Griseofulvin	126-07-8	Sigma Aldrich
Heroin	561-27-3	Sigma Aldrich (Cerilliant product)
Heroin D9	1338713-49-7	LGC standards (Middlesex, UK)
HMA	13062-61-8	Kinesis
HMMA	438625-58-2	Kinesis
HNE-MA	75899-68-2	Cayman Chemicals
Hydrocodone	125-29-1	Sigma Aldrich (Cerilliant product)
Hydrocodone D6	1007844-38-3	Sigma Aldrich (Cerilliant product)
Ibuprofen	15687-27-1	Sigma Aldrich
Ibuprofen D3	121662-14-4	Sigma Aldrich (Gillingham, UK)
Imatinib	152459-95-5	Sigma Aldrich
Imazalil sulphate	58594-72-2	Sigma Aldrich (Gillingham, UK)
Imidacloprid	138261-41-3	Sigma Aldrich (PESTANAL)
Indoprofen	31842-01-0	Sigma Aldrich (Gillingham, UK)
Iopromide	73334-07-3	LGC
Irbesartan	138402-11-6	LGC
Ketamine	1867-66-9	Sigma Aldrich
Ketamine D4	1246815-97-3	LGC standards (Middlesex, UK)
Ketoprofen	22071-15-4	Sigma Aldrich
Lisinopril	76547-98-3	LGC
Lomefloxacin	98079-52-8	Sigma Aldrich
MDA	101-77-9	LGC (Cerilliant product)
MDA D5	136765-42-9	LGC standards (Middlesex, UK)
MDMA	42542-10-9	LGC
MDMA D5	136765-43-0	LGC standards (Middlesex, UK)
MDPV	687603-66-3	Sigma Aldrich
Memantine	19982-08-2	Sigma Aldrich
Mephedrone	1189805-46-6	Sigma Aldrich (Cerilliant product)
Mephedrone D3	1189972-79-9	LGC standards (Middlesex, UK)
Metazachlor	67129-08-2	LGC (Dr Ehrenstorfer)
Metazachlor D6	1246816-51-2	Toronto Research Chemicals Inc. (Ontario, Canada)

Methadone	76-99-3	Sigma Aldrich (Cerilliant product)
Methadone D9	1435933-74-6	LGC standards (Middlesex, UK)
Methamphetamine	537-46-2	LGC (Cerilliant product)
Methamphetamine D5	60124-88-1	LGC standards (Middlesex, UK)
Methotrexate	59-05-2	LGC
Methylparaben	99-76-3	Sigma Aldrich
Methylparaben 13C6	1581694-95-2	LGC standards (Middlesex, UK)
Morphine	57-27-2	Sigma Aldrich (Cerilliant product)
Morphine D3	67293-88-3	LGC standards (Middlesex, UK)
Nalidixic acid	389-08-2	Sigma Aldrich
Naproxen	22204-53-1	Toronto Research Chemicals Inc. (Ontario, Canada)
N-desmethyltramadol	75377-45-6	LGC
N-guanylurea	207300-86-5	Sigma Aldrich
Nicotine	54-11-5	Sigma Aldrich
Norcodeine	467-15-2	Sigma Aldrich (Cerilliant product)
Nordiazepam	1088-11-5	Sigma Aldrich (Cerilliant product)
Nordiazepam D5	65891-80-7	Sigma Aldrich (Cerilliant product)
Norephedrine	154-41-6	Sigma Aldrich
Norfluoxetine	83891-03-6	LGC (Cerilliant product)
Normorphine	466-97-7	Sigma Aldrich (Cerilliant product)
Noroxycodone	52446-25-0	LGC
Nortriptyline	72-69-5	Sigma Aldrich
Nortriptyline D3	203784-52-5	Toronto Research Chemicals Inc. (Ontario, Canada)
O-6-MAM	2784-73-8	Sigma Aldrich (Cerilliant product)
O-desmethyltramadol	185453-02-5	LGC
O-desmethylnaproxen	52079-10-4	Sigma Aldrich (Gillingham, UK)
Ofloxacin	82419-36-1	Sigma Aldrich
Ofloxacin D3	82419-36-1	Toronto Research Chemicals Inc. (Ontario, Canada)
Omeprazole	73590-58-6	Sigma Aldrich (Gillingham, UK)
Orlistat	96829-58-2	Sigma Aldrich
Oxadiazon	19666-30-9	Sigma Aldrich (PESTANAL)
Oxycodone	76-42-6	Sigma Aldrich (Cerilliant product)
Oxycodone D6	152477-91-3	Sigma Aldrich (Cerilliant product)
Oxymorphone	76-41-5	Sigma Aldrich (Cerilliant product)
Penicillin G	61-33-6	Sigma Aldrich
Penicillin V	87-08-1	Sigma Aldrich
Pholcodine	509-67-1	Sigma Aldrich
PMA	3706-26-1	LGC
Praziquantrel	55268-74-1	Sigma Aldrich (Gillingham, UK)
Praziquantrel D11	1246343-36-1	LGC Standards (Teddington, UK)
Pregabalin	148553-50-8	LGC (Cerilliant product)
Propylparaben	94-13-3	Sigma Aldrich
Prulifloxacin	123447-62-1	Sigma Aldrich
Quetiapine	111974-69-7	LGC
Quetiapine D8	1185247-12-4	LGC standards (Middlesex, UK)
Ranitidine	66357-35-5	Sigma Aldrich
Risperidone	106266-06-2	LGC
Salbutamol	18559-94-9	Sigma Aldrich (Gillingham, UK)
Sarafloxacin	98105-99-8	Sigma Aldrich (VETRANAL)
Sertraline	79617-96-2	LGC
Sertraline D3	1217741-83-7	Sigma Aldrich (Gillingham, UK)
Sitagliptin	486460-32-6	TRC
Sotalol	3930-20-9	Sigma Aldrich (Gillingham, UK)
Sulphadiazine	68-35-9	Sigma Aldrich (VETRANAL)
Sulphamethoxazole	723-46-6	Sigma Aldrich
Sulphapyridine	144-83-2	Sigma Aldrich
Sulphasalazine	599-79-1	Sigma Aldrich
Terbutaline	23031-25-6	Sigma Aldrich (Gillingham, UK)
Terbuthylazine	5915-41-3	Sigma Aldrich
Tetramisole	5086-74-8	Sigma Aldrich (Gillingham, UK)
Tetramisole D5	1173021-85-6	LGC Standards (Teddington, UK)
Thiamethoxam	153719-23-4	LGC (Ultra)
Triallate	2303-17-5	Sigma Aldrich (PESTANAL)
Triclosan	3380-34-5	Sigma Aldrich
Valsartan	137862-53-4	Sigma Aldrich
Vardenafil	224789-1515-5	Sigma Aldrich (Cerilliant product)
Venlafaxine	93413-69-5	Sigma Aldrich
Zolpidem	99294-93-6	Sigma Aldrich (Cerilliant product)

Table S2. Method conditions selected for chiral separation

Method	Original column used	Mobile phase B	ABPR (PSI)	Flow rate (mL min^{-1})	Column Temp. (^oC)	Gradient conditions (total run time)	Make-up flow conditions	Ref
A	Amylose 3.0x150 mm, 2.5 µm	1:1 (v/v) IPA:EtOH	1990	2	35	0 min 3 % B, 4 min 30 % B, 6 min 30 % B (9 minutes)	98:2 (% v/v) MeOH/H_2O w/ 0.1 % NH_4OH @ 0.3 mL min^{-1}	(Twohig, O'Leary et al. 2014)
B	AMY-1, CEL-2	1:1:1 (v/v) MeOH:MeCN: IPA	1800	1.5	30	0 min 15 %B, 1 min 15 % B, 5 min 60 % B, 7 min 60 % B (9 minutes)	MeOH w/ 0.1 % formic acid @ 0.3 mL min^{-1}	(Camacho-Munoz, Kasprzyk-Hordern et al. 2016)
C		1:1 (v/v) EtOH:MeCN with 0.2 % (v/v) TFA	1800	1.5	30	0 min 5 % B, 3.5 min 5 % B, 10 min 60 % B, 13.5 min 60 % B (16 minutes)	MeOH w/ 0.2 % NH_4OH @ 0.3 mL min^{-1}	
D	CHIRAL PAK IB-3, 2.1x100 mm	20 mM NH_4OAc in MeOH	1500	2	35	0 min 7 % B, 1 min 7 % B, 7 min 25 % B, 9 min 25 % B (10 minutes)	Not given, conditions for method A used	(Waters 2012)

Table S3. Matrix of method conditions used to analyse analytes

SFC method / Column	AMY-1	CEL-1	CEL-2	Mobile phase conditions
A	A1	A2	A3	B: IPA:EtOH – 9 minute gradient
B	B1	B2	B3	B: MeOH:MeCN:IPA – 9 minute gradient
C	C1	C2	C3	B: EtOH:MeCN with 0.2 % TFA – 16 minute gradient
D	D1	D2	D3	B: MeOH with 20 mM NH_4OAC – 10 minute gradient

Table S1. Chiral separation using twelve different methods

Method / column	AMY-1	CEl-1	CEL-2
A	A1: Temazepam, tramadol (partial)	A2: Bisprolol, Metoprolol, propranolol, ketamine, Mepehdrone (partial), Methamphetamine (partial), MDMA (partial)	A:3 Temazepam
B	B1: Bisoprolol, temazepam, metoprolol, propranolol, ketamine, desmethylvenlafaxine, PMA (partial), Mephedrone (partial)	B:2 Bisprolol, metoprolol, atenolol, propranolol, temazepam (partially), PMA	B3: Bisoprolol, temazepam, atenolol, ketamine, propranolol (partial)
C	C1: Metoprolol, propranolol, EDDP	C2: Bisoprolol, temazepam, metoprolol (partially), atenolol, propranolol, ketamine, mephedrone (partially), fluoxetine	C3: Temazepam, atenolol
D	D1: Ketamine, methamphetamine (partial), Fluoxetine, betablockers separated but carry-over into the next injection	D2: Mephedrone and methamphetamine, MDMA (partial), PMA (partial)	D3: No compounds separated

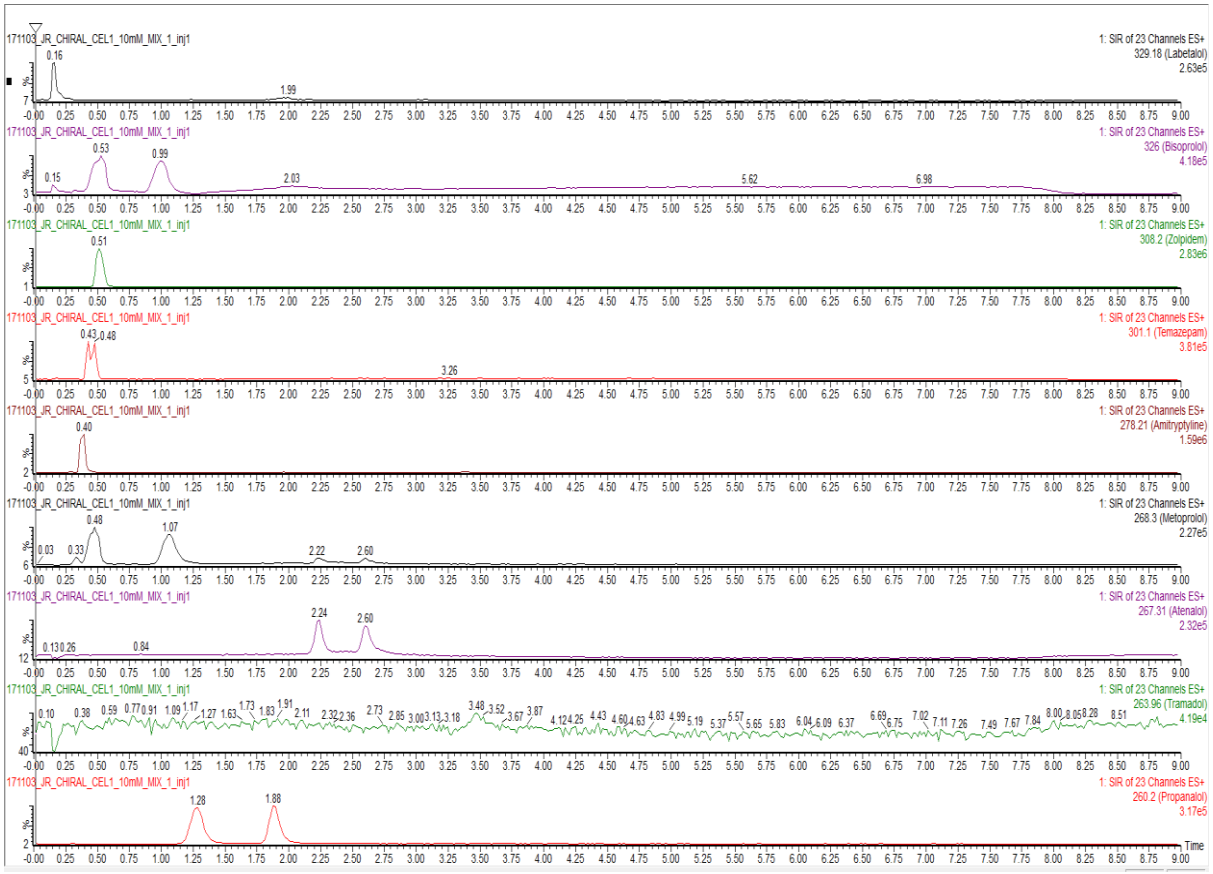


Figure S1. Enantioselective separation of bisoprolol, temazepam (partial), metoprolol, atenolol and propranolol in method B2. N.B. Labetolol elution occurred during column void volume

Table S5. MRM conditions of analytes and isotopically labelled internal standards studied using the selected SFC-TQD method

Analyte	MRM 1 transition	CV (V) / CE (V)	MRM 2 transition	CV (V) / CE (V)
1,7 dimethylxantine	181 > 124	54 / 21	-	-
1S,2R-(+)-ephedrine D3	169 > 151	23 / 18	-	-
2-Hydroxyibuprofen	221 > 177	30 /6	221 > 159	30 / 12
2-Phenylproponic Acid	149 > 105	20 / 5	-	-
8-isoF2B	353 > 309	53 / 18	353 > 247	53 / 22
Acetaminophen	152 > 110	26 / 16	152 > 93	26 / 24
Acetaminophen D4	156 > 114	26 / 16	-	-
AEME	182 > 118	39 / 23	182 > 122	39 /21
Aminorex	163 > 120	20 / 15	163 > 103	20 / 20
Amoxicillin	366 > 208	22 / 13	366 > 114	22 / 23
Amphetamine	136 > 119	18 / 8	136 > 91	18 / 16
Amphetamine D5	141 > 93	20 / 14	-	-
Ampicillin	350 > 160	44 / 10	350 > 107	44 / 28
Azathioprine	278 > 142	28 / 20	278 > 85	28 / 13
Azithromycin	750 > 116	60 / 54	750 > 83	60 / 60
Benzophenone-1	213 > 135	36 / 20	213 > 91	34 / 25
Benzophenone-2	245 > 109	32 / 20	245 > 135	32 / 13
Benzophenone-3	229 > 151	35 / 18	229 > 105	35 / 20
Benzophenone-4	307 > 227	44 / 24	307 > 211	42 / 35
Benzoylecgonine	290 > 168	38 / 19	290 > 105	38 / 30
Benzoylecgonine D8	298 > 171	38 / 19	-	-
Benzylpiperizine	177 > 91	35 / 20	177 > 85	35 / 15
Bezafibrate	360 > 274	30 / 19	360 > 154	30 / 28
Bicalutamide	431 > 217	40 / 15	431 > 187	40 / 13
Buprenorphine	468 > 396	66 / 41	468 > 414	66 / 35
Caffeine	195 > 138	38 / 15	195 > 110	38 / 23
Candesartan Cilexetil	611 > 567	44 / 7	611 > 467	44 / 7
Capecitabine	360 > 244	25 / 11	360 > 174	25 / 23
Carbamazepine	237 > 194	40 / 20	237 > 179	40 / 38
Carbamazepine-10,11-epoxide	253 > 210	39 / 12	253 > 180	39 / 25
Carbamazepine 13C6	243 > 200	40 / 20	-	-
Carboxyibuprofen	235 > 191	25 / 8	235 > 73	25 / 15
Carprofen	272 > 228	39 / 17	272 > 226	39 / 39
Cetirizine	389 > 201	32 / 21	389 > 166	32 / 40
Chloramphenicol	321 > 152	27 / 15	321 > 194	35 / 12
Chlorpyrifos	350 > 198	34 / 16	350 > 125	34 / 19
Cimetidine	253 > 159	22 / 16	253 > 211	22 / 10
Citalopram	325 > 262	46 / 18	325 > 110	46 / 26
Citalopram D6	331 > 109	46 / 28	-	-
Clarithromycin	749 > 590	40 / 20	749 > 158	40 / 31
Clothiniadin	250 > 132	28 / 15	250 > 113	28 / 25
Cocaethylene	318 > 196	38 / 20	318 > 82	38 / 30
Cocaethylene D3	321 > 199	40 / 22	-	-
Cocaine	304 > 182	40 / 20	304 > 82	40 / 31
Cocaine D3	307 > 185	40 / 20	-	-
Codeine	300 > 215	49 / 25	300 > 152	49 / 57
Codeine D6	306 > 218	52 / 28	-	-
Cotinine	177 > 80	34 / 21	177 > 98	34 / 22
Cotinine D3	180 > 80	44 / 24	-	-
Creatinine	114 > 44	30 / 15	114 > 86	31 / 11
Cytarabine	244 > 112	20 / 14	244 > 133	20 / 15
Danofloxacin	358 > 340	65 / 20	358 > 255	38 / 38
Desmethylcitalopram	311 > 109	46 / 27	311 > 262	46 / 18
Desmethylvenlafaxine	264 > 107	25 / 24	264 > 246	25 / 20
Desvenlafaxine	264 > 107	25 / 24	264 > 246	25/ 20
DHMA	182 > 123	6 / 18	182 > 151	6 / 12
Diazepam	285 > 154	56 / 29	285 > 222	56 / 27
Diazepam D5	290 > 198	56 / 34	-	-
Diazinon	305 > 169	36 / 22	305 > 153	36 / 22
Dichlofluanid	335 > 123	29 / 31	335 > 271	29 / 6
Diclofenac	294 > 250	22 / 13	-	-
Dihydrocodeine	302 > 199	53 / 33	302 > 128	53 / 60
Dihydroketoprofen	255 > 211	30 / 8	-	-
Dihydromorphine	288 > 185	28 / 42	288 > 213	28 / 32
Diltiazem	415 > 178	40 / 25	415 > 310	40 / 25
Donepezil	380 > 288	56 / 24	380 > 262	56 / 23

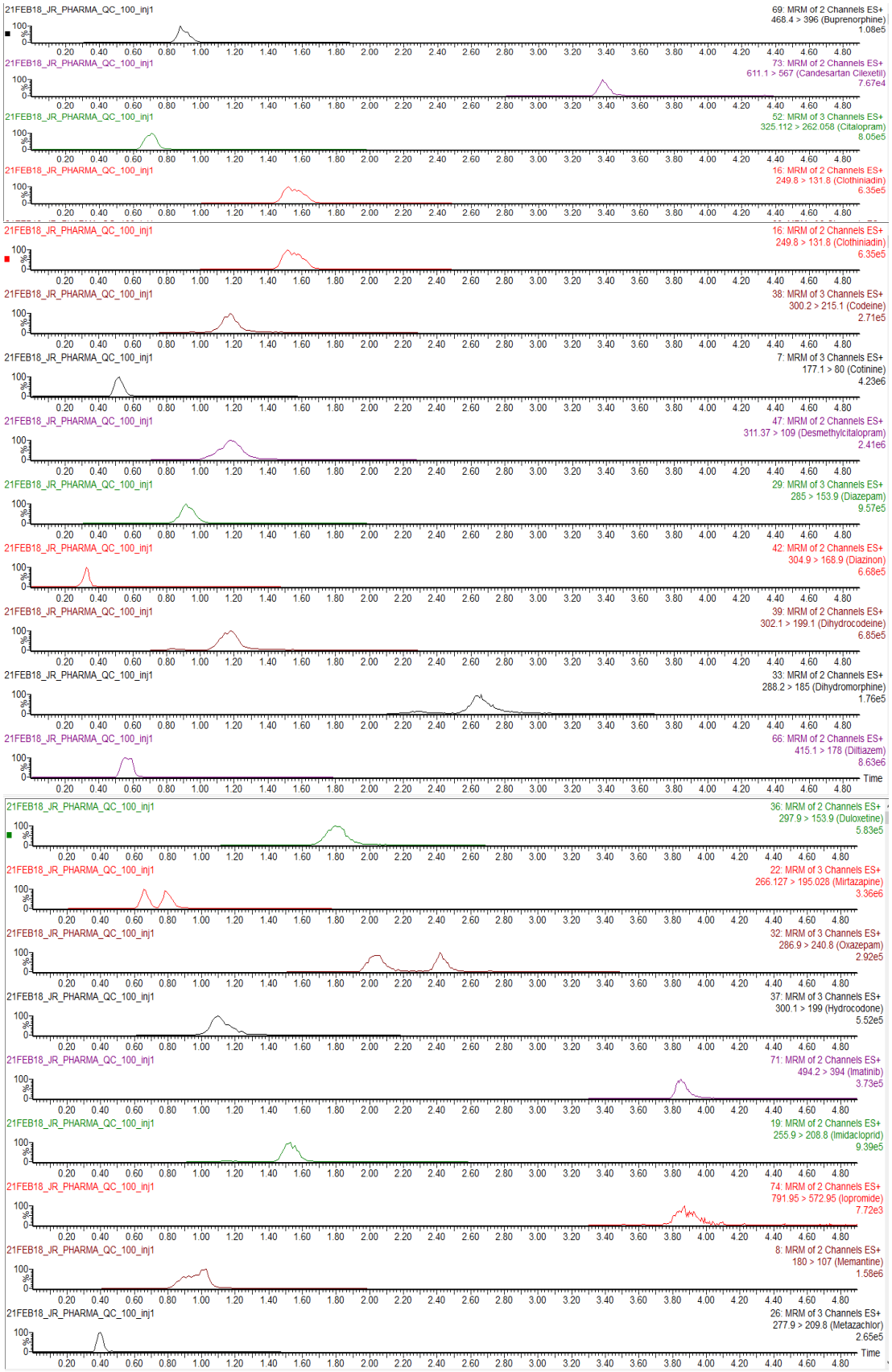
Duloxetine	298 > 154	16 / 5	280 > 188	16 / 5
E1 & E2-10,11-dihydro-10-hydroxycarbamazepine	255 > 194	20 / 20	255 > 179	20 / 40
E1 & E2-Alprenolol	250 > 116	44 / 16	250 > 98	44 / 18
E1 & E2-Atenolol	267 > 145	38 / 30	267 > 190	38 / 16
E1 & E2-Atenolol D7	274 > 145	44 / 30	-	-
E1 & E2-Bisoprolol	326 > 116	45 / 18	326 > 204	45 / 19
E1 & E2-Metoprolol	268 > 116	42 / 20	268 > 121	42 / 22
E1 & E2-Mirtazapine	266 > 195	44 / 18	266 > 72	44 / 26
E1 & E2-Mirtazapine D3	269 > 195	35 / 25	-	-
E1 & E2-Propanolol	260 > 116	42 / 16	260 > 183	42 / 18
E1 & E2-Tramadol	264 > 58	28 / 45	264 > 121	28 / 46
E1& E2-Metoprolol D7	275 > 123	44 / 20	-	-
E1& E2-Oxazepam	287 > 241	38 / 22	287 > 269	38 /14
E1& E2-Oxazepam D5	292 > 274	38 / 15	-	-
E1& E2-Propanolol D7	267 > 189	40 / 18	-	-
Ecgonine methylester D3	203 > 85	44 / 22	-	-
EE2	295 > 159	60 / 40	295 > 145	60 / 40
Ephedrine	166 > 148	23 / 12	166 > 133	23 / 21
Erythromycin	735 > 159	12 / 32	735 > 576	12 / 20
Ethylparaben	165 > 92	26 / 20	164 > 137	20 / 14
Fexofenadine	500 > 456	33 / 14	500 > 378	33 / 19
Flufenacet	364 > 194	24 / 10	364 > 152	24 / 18
Flumequine	262 > 202	28 / 34	262 > 245	28 / 26
Fluoxetine	310 > 44	34 / 10	310 > 148	34 / 10
Fluoxetine D5	315 > 153	26 / 8	-	-
Furosemide	331 > 313	31 / 8	331 > 239	31 / 10
Gabapentin	172 > 154	30 / 12	172 > 95	30 / 22
Gabapentin D4	176 > 158	33 / 16	-	-
Gemfibrozil	251 > 205	21 / 9	251 > 123	21 / 14
Gliclazide	324 > 127	41 / 20	324 > 110	41 / 20
Griseofulvin	353 > 69	45 / 25	353 > 165	45 / 23
Heroin	370 > 165	51 / 50	370 > 268	51 / 29
Heroin D9	379 > 166	51 / 50	-	-
HMA	182 > 123	6 / 18	182 > 165	6 / 14
HMMA	196 > 165	16 / 12	196 > 133	16 / 22
HNE-MA	318 > 171	32 / 22	318 > 162	32 / 14
Hydrocodone	300 > 199	24 / 34	300 > 171	24 / 46
Hydrocodone D6	306 > 202	64 / 32	-	-
Ibuprofen	205 > 161	19 / 8	-	-
Ibuprofen D3	208 > 164	20 / 6	-	-
Imatinib	494 > 394	57 / 27	494 > 378	57 / 48
Imazalil sulphate	297 > 159	40 / 20	297 > 201	40 / 18
Imidacloprid	256 > 209	34 / 15	256 > 175	34 / 19
Indoprofen	282 > 236	45 / 20	282 > 77	45 / 15
Iopromide	792 > 573	46 / 25	792 > 559	46 / 32
Irbesartan	427 > 193	50 / 28	427 > 121	50 / 65
Ketamine	238 > 125	31 / 27	238 > 220	31 / 15
Ketamine D4	242 > 129	31 / 27	-	-
Ketoprofen	253 > 209	15 / 7	253 > 212	15 / 7
Lisinopril	406 > 84	38 / 27	406 > 246	38 / 22
Lomefloxacin	352 > 265	22 / 24	352 > 308	22 / 22
MDA	180 > 163	21 / 11	180 > 105	21 / 22
MDA D5	185 > 168	21 / 11	-	-
MDMA	194 > 163	24 / 13	194 > 105	24 / 24
MDMA D5	199 > 165	26 /13	-	-
MDPV	276 > 126	40 / 28	276 > 135	40 / 25
Memantine	180 > 107	36 / 24	180 > 121	36 / 24
Mephedrone	178 > 145	10 / 22	178 > 160	10 / 12
Mephedrone D3	181 > 148	30 / 22	-	-
Metazachlor	278 > 210	21 / 21	278 > 134	21 / 10
Metazachlor D6	284 > 216	21 / 10	-	-
Methadone	310 > 265	31 / 15	310 > 105	31 /28
Methadone D7	319 > 268	31 / 15	-	-
Methamphetamine	150 > 91	24 / 19	150 > 119	24 / 10
Methamphetamine D5	155 > 92	28 / 18	-	-
Methotrexate	455 > 175	40 / 35	455 > 308	40 / 20
Methylparaben	151 > 92	34 / 20	151 > 136	20 / 14
Methylparaben 13C6	157 > 98	30 / 20	-	-
Morphine	286 > 165	53 / 38	286 > 152	53 / 56
Morphine D3	289 > 152	53 / 56	-	-
Nalidixic acid	233 > 187	30 / 28	233 > 131	30 / 36

Naproxen	229 > 169	20 / 8	229 > 185	20 / 8
N-desmethyl tramadol	250 > 44	25 / 12	250 > 232	25 / 8
N-Guanylurea	103 > 60	24 / 10	103 > 86	24 / 8
Nicotine	163 > 130	37 / 20	163 > 117	37 / 24
Norcodeine	286 > 165	46 / 40	286 > 268	46 / 20
Nordiazepam	271 > 140	51 / 29	271 > 165	51 / 29
Nordiazepam D5	276 > 140	48 / 36	-	-
Norephedrine	152 > 134	23 / 10	152 > 117	23 / 16
Norfluoxetine	296 > 134	18 / 6	-	-
Normorphine	272 > 165	45 / 43	272 > 152	45 / 49
Noroxycodone	302 > 227	22 / 36	302 > 187	22 /38
Nortriptyline	264 > 91	33 / 23	264 > 233	33 / 16
Nortriptyline D3	267 > 191	40 / 20	267 > 233	40 / 8
O-6-MAM	328 > 165	52 / 39	328 > 211	52 / 26
O-desmethyl tramadol	250 > 58	30 > 18	250 > 232	30 / 10
O-Desmethylnaproxen	215 > 170	20 / 10	-	-
Ofloxacin	362 > 261	43 / 28	362 > 318	43 / 19
Ofloxacin D3	365 > 261	47 / 28	365 > 322	47 / 23
Omeprazole	346 > 198	20 / 10	-	-
Orlistat	496 > 319	40 / 13	496 > 160	40 / 12
Orlistat	496 > 319	40 / 13	496 > 160	40 / 12
Oxadiazon	345 > 303	43 / 14	344.9 > 302.8	43 / 20
Oxycodone	316 > 241	36 / 29	316 > 256	36 / 26
Oxycodone D6	322 > 247	36 / 29	-	-
Oxymorphone	302 > 284	40 / 19	302 > 227	40 / 28
Penicillin G	335 > 176	48 / 20	335 > 160	48 / 20
Penicillin V	316 > 114	54 / 40	316 > 160	54 / 40
Pholcodine	399 > 381	55 / 25	399 > 100	55 / 35
PMA	166 > 121	20 / 20	166 > 149	20 / 20
Praziquantrel	313 > 203	40 / 15	313 > 83	40 / 35
Praziquantrel D11	325 > 204	40 / 20	-	-
Pregabalin	160 > 142	32 / 11	160 > 125	32 / 14
Propylparaben	179 > 92	34 / 25	179 > 136	20 / 16
Prulifloxacin	462 > 444	42 / 22	462 > 360	42 / 32
Quetiapine	384 > 253	50 / 21	384 > 221	50 / 40
Quetiapine D8	392 > 258	50 / 23	-	-
Ranitidine	316 > 176	26 / 17	316 > 124	26 / 14
Risperidone	411 > 191	49 / 30	411 > 110	49 / 51
Salbutamol	240 > 148	30 / 18	240 > 166	30 / 14
Sarafloxacin	386 > 368	49 / 23	386 > 299	49 / 28
Sertraline	306 > 159	23 / 27	306 > 275	23 / 10
Sertraline D3	309 > 159	23 / 27	-	-
Sitagliptin	408 > 235	46 / 19	408 > 193	46 / 26
Sotalol	273 > 133	30 / 28	273 > 213	30 / 16
Sulphadiazine	251 > 108	30 / 26	251 > 158	30 / 15
Sulphamethoxazole	254 > 92	36 / 30	254 > 156	36 / 20
Sulphapyridine	250 > 156	42 / 16	250 > 92	42 / 30
Sulphasalazine	397 > 197	45 / 25	397 > 240	45 / 25
Terbutaline	226 > 105	66 / 22	-	-
Terbuthylazine	230 > 174	35 / 17	230 > 132	35 / 24
Tetramisole	205 > 91	45 / 35	205 > 123	45 / 30
Tetramisole D5	211 > 183	30 / 15	-	-
Thiamethoxam	292 > 211	44 / 12	292 > 132	44 / 22
Triallate	306 > 145	34 / 26	306 > 128	34 / 13
Triclosan	289 > 35	18 / 10	289 > 37	18 / 10
Valsartan	434 > 350	35 / 20	434 > 179	35 / 25
Vardenafil	489 > 151	74 / 68	489 > 312	74 / 48
Venlafaxine	278 > 58	27 / 40	278 > 260	27 / 12
Zolpidem	308 > 221	8 / 44	-	-

Table S*6*. Average analyte retention times in each of the three methods (n=42).

PHARMA method analyte	t_R (min)	SD	DAC method analyte	t_R (min)	SD	NEG method analyte	t_R (min)	SD
1,7-DMX	0.8	0.01	AEME Ecgoninemethylester D3	0.3	0.0	2-Hydroxy-ibuprofen	1.1	0.04
Acetaminophen Acetaminophen D4	0.8	0.02	Aminorex	1.3	0.03	2-Phenylproponic acid	N/A	0.03
Azathioprine	2.5	0.02	Amoxicillin	3.4	0.05	8-IsoF2B	2.7	0.02
Benzophenone-3	0.4	0.01	Amphetamine Amphetamine D5	0.8	0.00	Benzophenone-1	0.6	0.01
Bicalutamide	0.7	0.02	Ampicillin	2.9	0.05	Benzophenone-2	N/A	N/A
Buprenorphine	0.9	0.01	Azithromycin	1.2	0.05	Benzophenone-4	4.5	0.01
Caffeine	0.6	0.00	Benzoylecgonine Benzoylecgonine D8	2.4	0.01	Bezafibrate	3.8	0.01
Candesartan Cilexetil	3.4	0.01	Benzylpiperizine	1.0	0.01	Carboxyibuprofen	4.0	0.04
Capecitabine	1.4	0.02	Carbamazepine Carbamazepine $^{13}C_6$	1.6	0.00	Carprofen	3.1	0.02
Chlorpyrifos	0.4	0.01	Carbamazepine-10,11-epoxide	2.1	0.01	Chloramphenicol	1.0	0.01
Cimetidine	2.2	0.03	Cetirizine	3.2	0.05	Diclofenac	2.4	0.01
Citalopram Citalopram D6	0.7	0.01	Clarithromycin	1.2	0.05	Dihydro-ketoprofen	2.5	0.02
Clothiniadin	1.5	0.02	Cocaethylene Cocaethylene D3	0.4	0.00	EE2	N/A	N/A
Codeine Codeine D6	1.2	0.01	Cocaine Cocaine D3	0.4	0.00	Ethylparaben	0.4	0.01
Cotinine Cotinine D3	0.5	0.00	Danofloxacin	3.0	0.05	Fexofenadine	4.2	0.02
Creatinine	2.0	0.04	DHMA	0.4	0.01	HNE-MA	3.9	0.02
Cytarabine	3.6	0.02	E1-10,11-dihydro-10-hydroxy-carbamazepine	1.4	0.01	Ibuprofen Ibuprofen D_3	0.5	0.02
Desmethyl-citalopram	1.2	0.01	E1-Alprenolol	0.8	0.01	Irebsartan	3.4	0.00
Desmethyl-venlafaxine	1.1	0.01	E1-Atenolol E1-Atenolol D7	3.1	0.01	Ketoprofen	1.2	0.02
Desvenlafaxine	1.1	0.01	E1-Bisoprolol	1.1	0.00	Methylparaben Methylparaben $^{13}C_6$	0.4	0.01
Diazepam Diazepam D5	0.9	0.01	E1-Metoprolol E1-Metoprolol D7	1.0	0.02	Naproxen	N/A	N/A
Diazinon	0.3	0.00	E1-Propanolol E1-Propanolol D7	2.3	0.01	O-desmethyl-naproxen	2.9	0.01
Dichlofluanid	0.4	0.01	E1-Tramadol	0.6	0.00	Propylparaben	0.4	0.01
Dihydrocodeine	1.2	0.01	E2-10,11-dihydro-10-hydroxy-carbamazepine	1.8	0.01	Sulphasalazine	5.5	0.02
Dihydromorphine	2.6	0.02	E2-Alprenolol	1.1	0.01	Triclosan	0.6	0.01
Diltiazem	0.6	0.00	E2-Atenolol E2-Atenolol D7	3.4	0.00	Valsartan	7.1	0.04
Duloxetine	1.8	0.01	E2-Bisoprolol	2.0	0.01			
E1-Mirtazapine E1-Mirtazapine D3	0.7	0.01	E2-Metoprolol E2-Metoprolol D7	2.1	0.01			
E1-Oxazepam E1-Oxazepam D5	2.0	0.01	E2-Propanolol E2-Propanolol D7	2.8	0.01			
E2-Mirtazapine E2-Mirtazapine D3	0.8	0.01	E2-Tramadol	1.1	0.00			
E2-Oxazepam E2-Oxazepam D5	2.4	0.01	Ephedrine	1.0	0.02			
Flufenacet	0.4	0.01	Erythromycin	0.9	0.05			
Fluoxetine Fluoxetine D5	0.6	0.01	Flumequine	1.5	0.05			
Furosemide	0.5	0.01	Griseofulvin	0.8	0.00			
Gabapentin Gabapentin D4	3.6	0.02	Heroin Heroin D9	0.5	0.01			
Gemfibrozil	0.4	0.02	HMA	2.1	0.01			
Gliclazide	1.0	0.02	HMMA	1.7	0.01			
Hydrocodone Hydrocodone D6	1.1	0.02	Imazalil sulphate	0.6	0.02			
Imatinib	3.8	0.01	Indoprofen	2.7	0.01			
Imidacloprid	1.5	0.01	Ketamine Ketamine D4	0.5	0.00			
Iopromide	3.9	0.03	Lomefloxacin	3.2	0.05			
Memantine	1.0	0.01	MDA MDA D5	1.0	0.03			
Metazachlor	0.4	0.01	MDMA MDMA D5	0.8	0.01			
Morphine Morphine D3	2.2	0.02	MDPV	0.4	0.01			

N-guanylurea	2.3	0.02	Mephedrone Mephedrone D3	0.4	0.01
Nicotine	0.4	0.01	Methadone Methadone D7	0.6	0.00
Norcodeine	2.8	0.02	Methamphetamine Methamphetamine D5	0.7	0.00
Nordiazepam Nordiazepam D5	1.5	0.01	Nalidixic acid	1.0	0.05
Norfluoxetine	1.0	0.01	N-desmethyl-tramadol	1.2	0.03
Normorphine	3.5	0.02	Norephedrine	1.4	0.01
Noroxycodone	2.7	0.03	O-6-MAM	1.0	0.02
Nortriptyline Nortryptyline D3	1.3	0.02	O-desmethyl-tramadol	1.1	0.01 0.01
Omeprazole	1.2	0.01	Ofloxacin Ofloxacin D3	2.7	0.01
Orlistat	0.4	0.01	Orlistat	0.5	0.02
Oxadiazon	0.3	0.01	Penicillin G	3.4	0.05
Oxycodone Oxycodone D6	0.7	0.01	Penicillin V	3.0	0.05
Oxymorphone	2.7	0.02	PMA	0.9	0.02
Pholcodine	2.2	0.01	Praziquantrel Praziquantrel D11	1.0	0.01
Pregabalin	3.6	0.02	Prulifloxacin	3.4	0.05
Quetiapine Quetiapine D8	1.3	0.01	Sarafloxacin	3.2	0.05
Ranitidine	2.6	0.01	Sulphadiazine	2.4	0.00
Risperidone	1.9	0.01	Sulphamethoxazole	1.8	0.01
Salbutamol	3.0	0.02	Sulphapyridine	2.3	0.00
Sertraline Sertraline D3	1.1	0.01	Tetramisole Tetramisole D5	0.8	0.00
Sitagliptin	1.4	0.03	Tylosin	N/A	N/A
Sotalol	2.5	0.01			
Terbutaline	0.6	0.00			
Terbuthylazine	0.4	0.00			
Thiamethoxam	1.4	0.02			
Triallate	0.4	0.02			
Vardenafil	3.2	0.02			
Venlafaxine	0.5	0.00			
Zolpidem	1.0	0.01			


21FEB18_JR_PHARMA_QC_100_inj1
69: MRM of 2 Channels ES+
468.4 > 396 (Buprenorphine)
1.08e5
73: MRM of 2 Channels ES+
611.1 > 567 (Candesartan Cilexetil)
7.67e4
52: MRM of 3 Channels ES+
325.112 > 262.058 (Citalopram)
8.05e5
16: MRM of 2 Channels ES+
249.8 > 131.8 (Clothiniadin)
6.35e5
38: MRM of 3 Channels ES+
300.2 > 215.1 (Codeine)
2.71e5
7: MRM of 3 Channels ES+
177.1 > 80 (Cotinine)
4.23e6
47: MRM of 2 Channels ES+
311.37 > 109 (Desmethylcitalopram)
2.41e6
29: MRM of 3 Channels ES+
285 > 153.9 (Diazepam)
9.57e5
42: MRM of 2 Channels ES+
304.9 > 168.9 (Diazinon)
6.68e5
39: MRM of 2 Channels ES+
302.1 > 199.1 (Dihydrocodeine)
6.85e5
33: MRM of 2 Channels ES+
288.2 > 185 (Dihydromorphine)
1.76e5
66: MRM of 2 Channels ES+
415.1 > 178 (Diltiazem)
8.63e6
36: MRM of 2 Channels ES+
297.9 > 153.9 (Duloxetine)
5.83e5
22: MRM of 3 Channels ES+
266.127 > 195.028 (Mirtazapine)
3.36e6
32: MRM of 3 Channels ES+
286.9 > 240.8 (Oxazepam)
2.92e5
37: MRM of 3 Channels ES+
300.1 > 199 (Hydrocodone)
5.52e5
71: MRM of 2 Channels ES+
494.2 > 394 (Imatinib)
3.73e5
19: MRM of 2 Channels ES+
255.9 > 208.8 (Imidacloprid)
9.39e5
74: MRM of 2 Channels ES+
791.95 > 572.95 (Iopromide)
7.72e3
8: MRM of 2 Channels ES+
180 > 107 (Memantine)
1.58e6
26: MRM of 3 Channels ES+
277.9 > 209.8 (Metazachlor)
2.65e5
Time

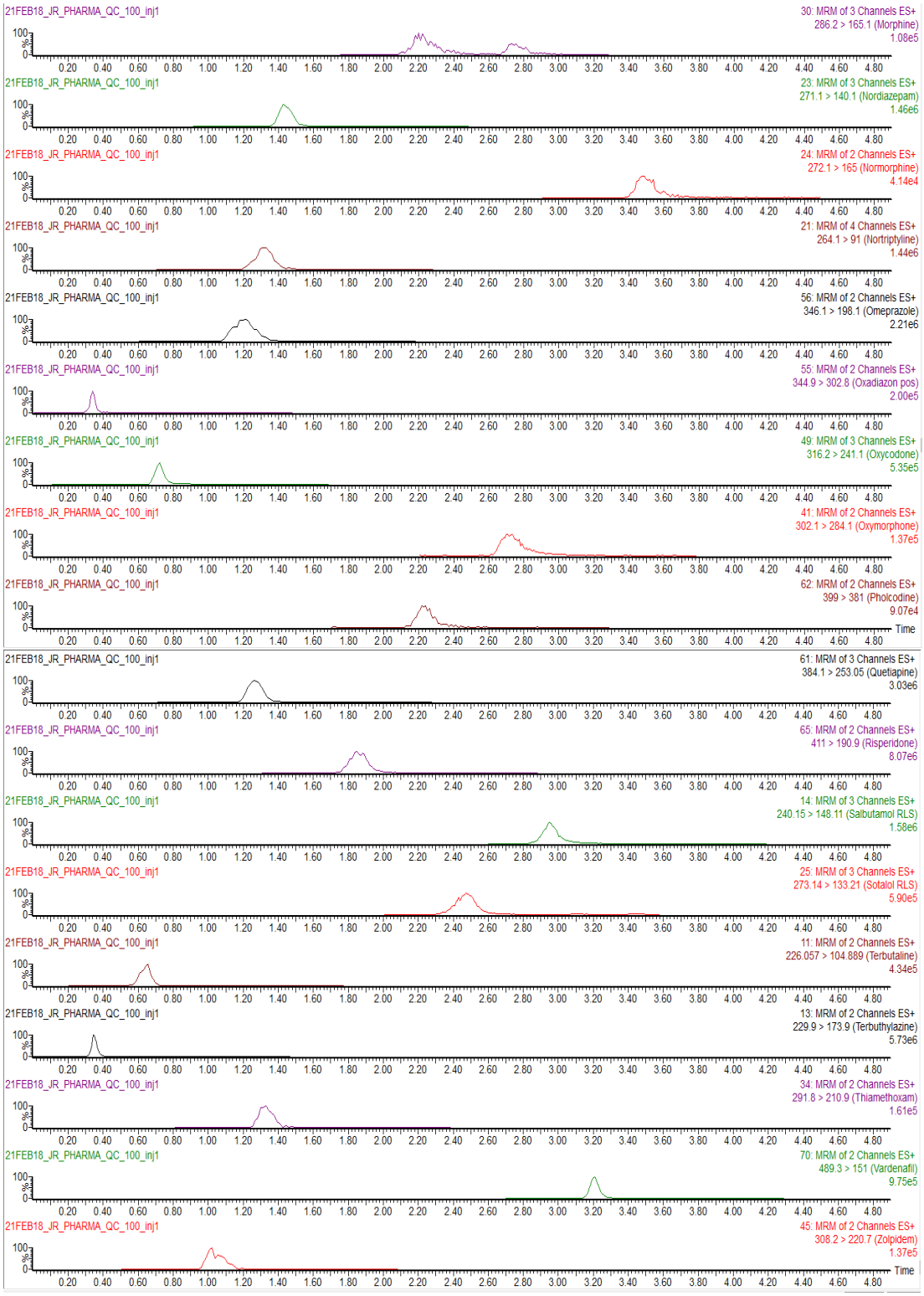
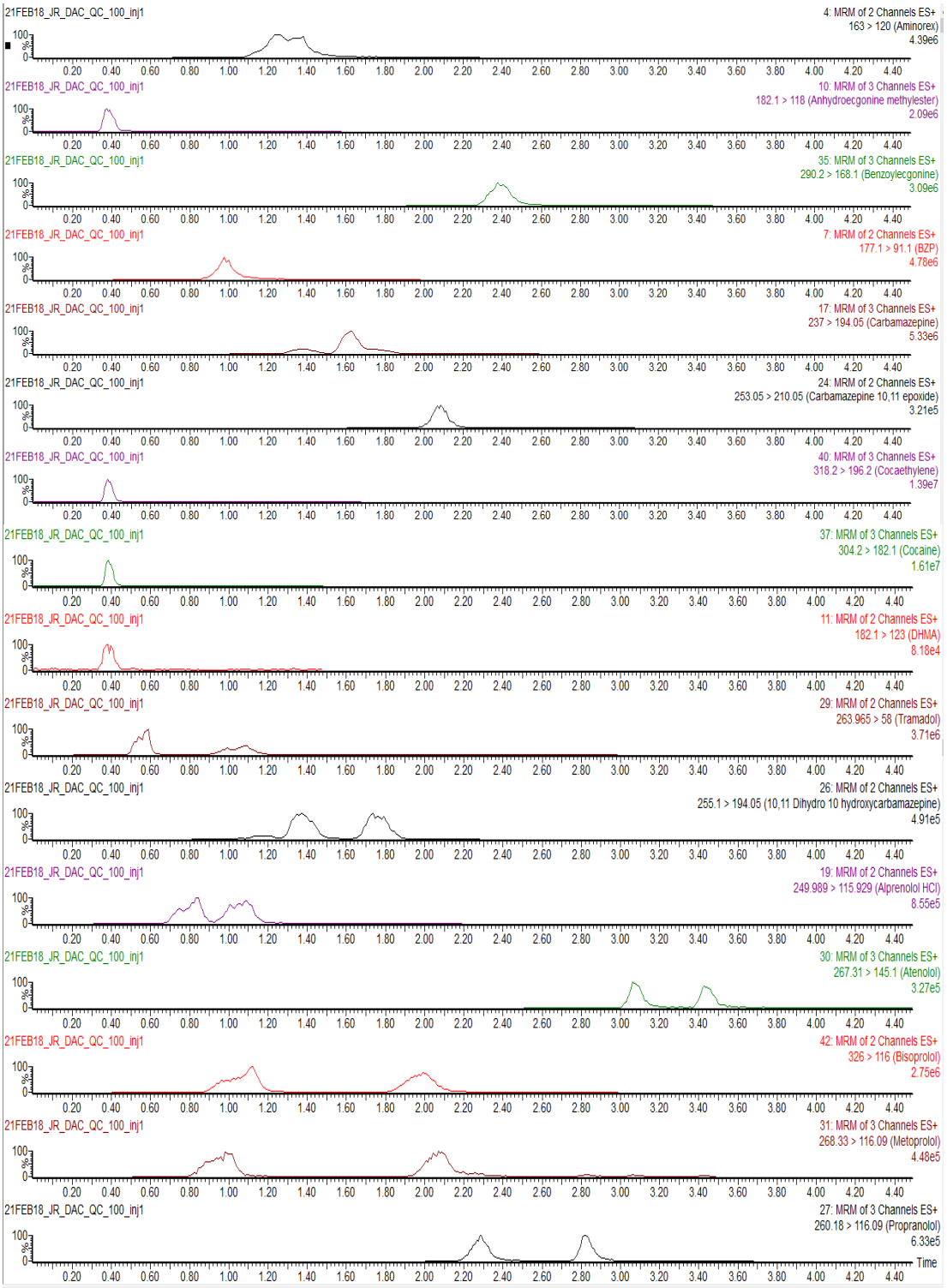


Figure S2. Extracted mass chromatograms for analytes in the PHARMA method using their MRM 1 transitions


21FEB18_JR_DAC_QC_100_inj1
4: MRM of 2 Channels ES+
163 > 120 (Aminorex)
4.39e6
10: MRM of 3 Channels ES+
182.1 > 118 (Anhydroecgonine methylester)
2.09e6
35: MRM of 3 Channels ES+
290.2 > 168.1 (Benzoylecgonine)
3.09e6
7: MRM of 2 Channels ES+
177.1 > 91.1 (BZP)
4.78e6
17: MRM of 3 Channels ES+
237 > 194.05 (Carbamazepine)
5.33e6
24: MRM of 2 Channels ES+
253.05 > 210.05 (Carbamazepine 10,11 epoxide)
3.21e5
40: MRM of 3 Channels ES+
318.2 > 196.2 (Cocaethylene)
1.39e7
37: MRM of 3 Channels ES+
304.2 > 182.1 (Cocaine)
1.61e7
11: MRM of 2 Channels ES+
182.1 > 123 (DHMA)
8.18e4
29: MRM of 2 Channels ES+
263.965 > 58 (Tramadol)
3.71e6
26: MRM of 2 Channels ES+
255.1 > 194.05 (10,11 Dihydro 10 hydroxycarbamazepine)
4.91e5
19: MRM of 2 Channels ES+
249.989 > 115.929 (Alprenolol HCl)
8.55e5
30: MRM of 3 Channels ES+
267.31 > 145.1 (Atenolol)
3.27e5
42: MRM of 2 Channels ES+
326 > 116 (Bisoprolol)
2.75e6
31: MRM of 3 Channels ES+
268.33 > 116.09 (Metoprolol)
4.48e5
27: MRM of 3 Channels ES+
260.18 > 116.09 (Propranolol)
6.33e5
Time

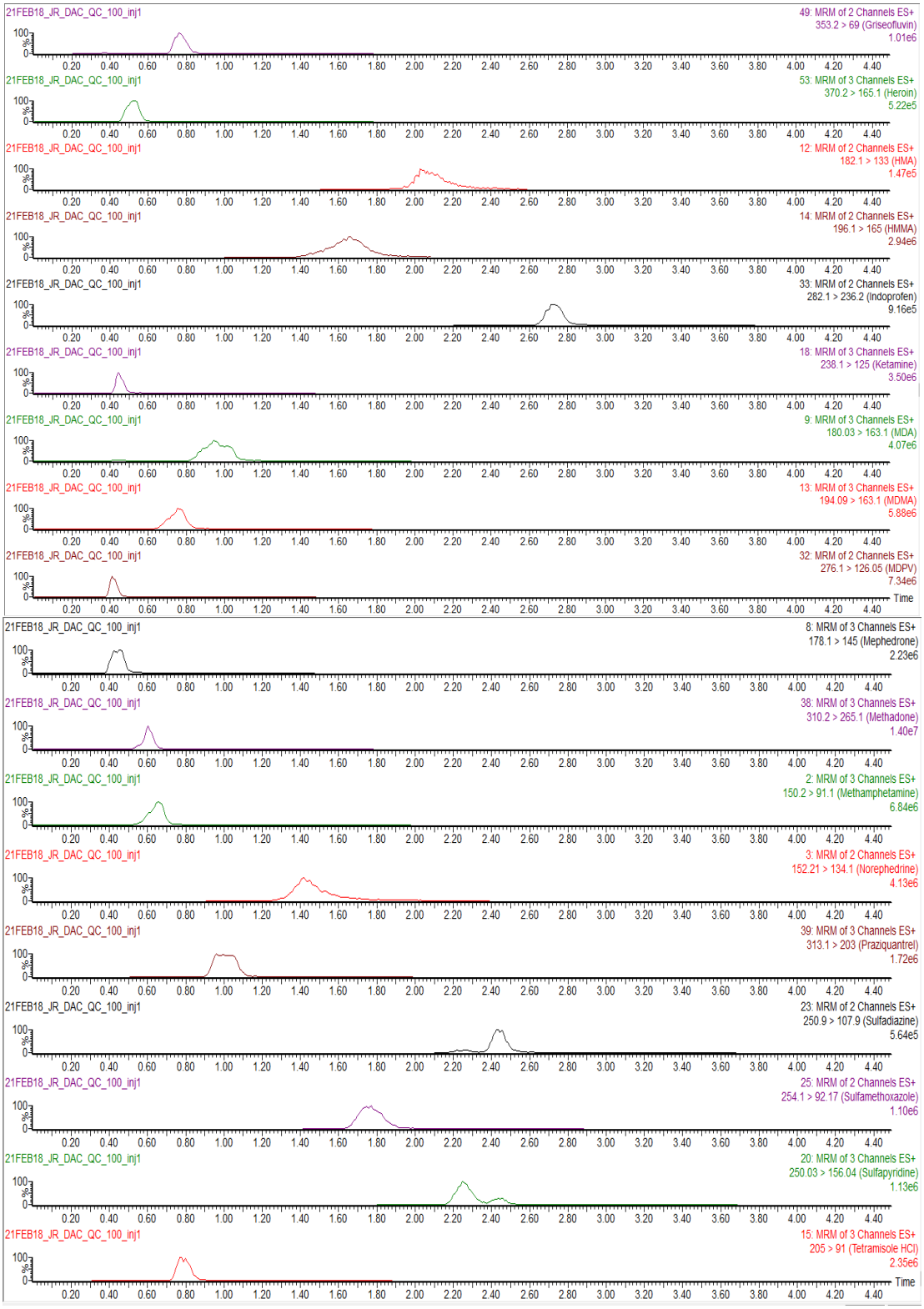


Figure S3. Extracted mass chromatograms for analytes in the DAC method using their MRM 1 transitions

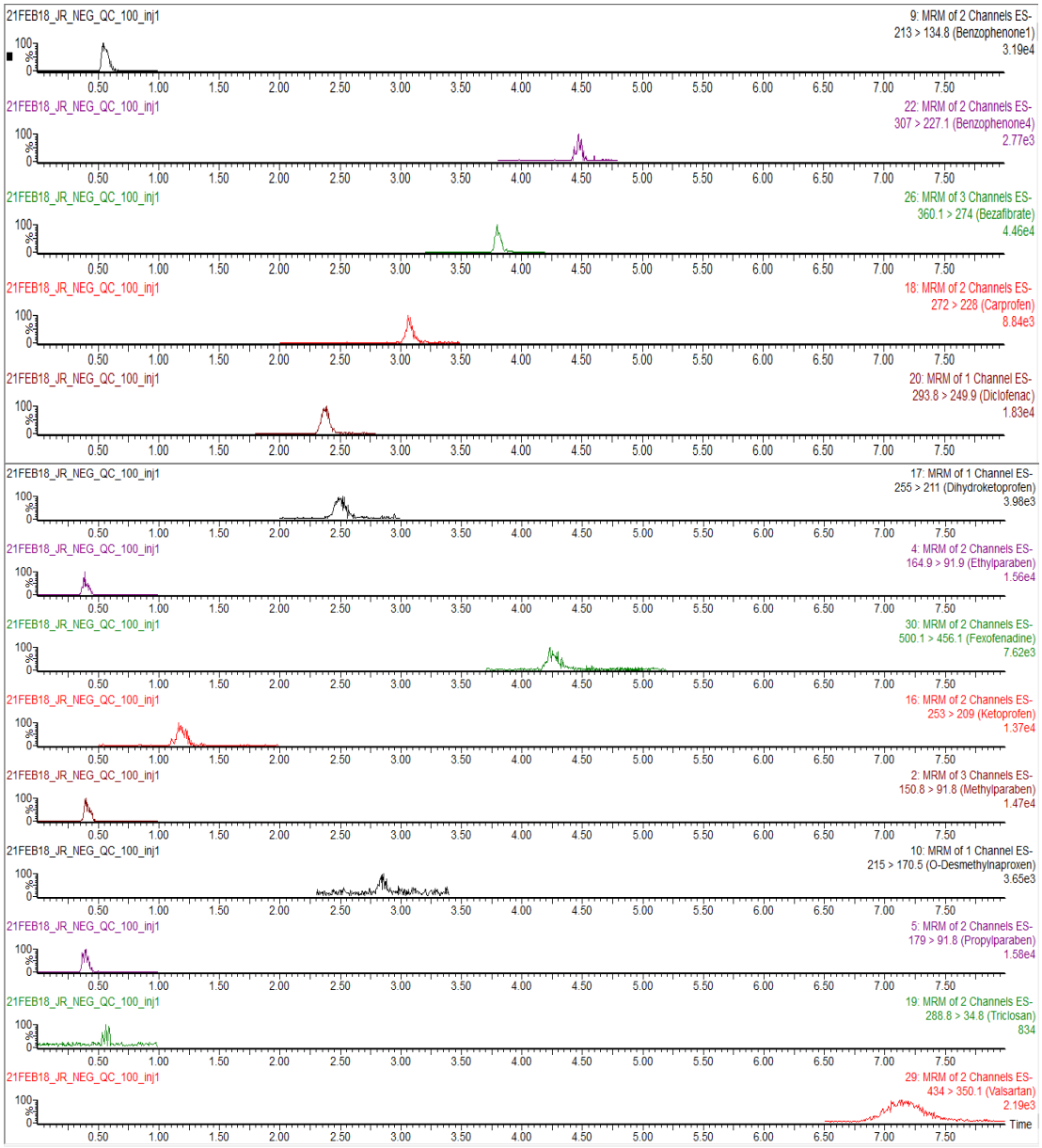


Figure S4. Mass chromatograms for analytes in the NEG method using their MRM1 transitions

Table S7. Instrument linearity, range, instruments limits of detection and quantification and average relative retention time (t_{rel}) (n=42; semiquantitative compounds are presented in italics)

Analyte	Average t_{rel}	Linear range (µg L^{-1})	R^2	iLOD (µg L^{-1})	iLOQ (µg L^{-1})	Assigned internal standard
Aminorex	1.6±0.02	1.5-600	0.997	0.50	1.50	Tetramisole D5
Anhydroecgonine methylester	1.0±0.02	0.1-600	0.999	0.05	0.10	Ecgonine methylester D3
Benzophenone-1	1.1±0.03	5-1000	0.998	1.00	5.00	Ibuprofen D3
Benzophenone-4	11.1±0.02	15-800	0.994	5.00	15.00	Methylparaben 13C6
Benzoylecgonine	1.0±0.003	0.05-600	0.998	0.01	0.05	Benzoylecgonine D8
Benzylpiperizine	*7.5±0.02*	*0.5-800*	*0.999*	*0.10*	*0.50*	*Amphetamine D5*
Bezafibrate	1.1±0.02	5-1000	0.997	1.00	5.00	Ibuprofen D3
Buprenorphine	1.2±0.01	5-400	0.999	1.00	5.00	E2-Mirtazapine D3
Candesartan Cilexetil	*0.9±0.01*	*5-400*	*0.999*	*1.00*	*5.00*	*Gabapentin D4*
Carbamazepine	1.0±0.004	0.5-600	0.999	0.10	0.50	Carbamazepine 13C6
Carbamazepine 10,11 epoxide	1.3±0.01	0.5-600	0.998	0.10	0.50	Carbamazepine 13C6
Carprofen	*6.1±0.03*	*25-600*	*0.997*	*10.00*	*25.00*	*Ibuprofen D3*
Citalopram	0.9±0.02	1.5-400	0.998	0.50	1.50	Citalopram D6
Clothiniadin	1.7±0.01	0.5-400	0.998	0.10	0.50	Diazepam D5
Cocaethylene	1.0±0.01	0.15-600	0.999	0.05	0.15	Cocaethylene D3
Cocaine	1.0±0.02	0.15-600	0.998	0.05	0.15	Cocaine D3
Codeine	1.0±0.01	0.15-400	0.998	0.05	0.15	Codeine D6
Cotinine	1.0±0.01	0.15-200	0.999	0.05	0.15	Cotinine D3
Desmethylcitalopram	1.0±0.01	0.5-400	0.998	0.10	0.50	Codeine D6
DHMA	*0.5±0.03*	*25-600*	*0.993*	*10.00*	*25.00*	*MDMA D5*
Diazepam	1.0±0.01	0.15-200	0.999	0.05	0.15	Diazepam D5
Diazinon	0.2±0.01	1.5-600	0.999	0.50	1.50	E1-Oxazepam D5
Diclofenac	4.7±0.03	5-600	0.997	1.00	5.00	Ibuprofen D3
Dihydrocodeine	1.0±0.01	0.15-200	0.997	0.05	0.15	Codeine D6
Dihydroketoprofen	4.9±0.03	25-1000	0.995	10.00	25.00	Ibuprofen D3
Dihydromorphine	1.2±0.01	5-400	0.998	1.00	5.00	Morphine D3
Diltiazem	*0.8±0.01*	*0.5-800*	*0.999*	*0.10*	*0.50*	*Oxycodone D6*
Duloxetine	*4.5±0.01*	*0.15-600*	*0.997*	*0.05*	*0.15*	*Metazachlor D6*
E1-10,11-dihydro-10-hydroxycarbamazepine	0.8±0.01	0.5-200	0.998	0.10	0.5	Carbamazepine 13C6
E1-Alprenolol	0.3±0.01	0.25-200	0.997	0.05	0.25	E1-Atenolol D7
E1-Atenolol	1.0±0.003	0.75-600	0.998	0.25	0.75	E1-Atenolol D7
E1-Bisoprolol	1.1±0.01	0.25-200	0.999	0.05	0.25	E1-Metoprolol D7
E1-Metoprolol	1.0±0.02	0.5-200	0.999	0.25	0.5	E1-Metoprolol D7
E1-Mirtazapine	1.0±0.01	0.25-100	0.998	0.05	0.25	E1-Mirtazapine D3
E1-Oxazepam	1.0±0.01	0.5-200	0.998	0.01	0.50	E1-Oxazepam D5
E1-Propanolol	1.0±0.004	1.5-600	0.998	0.50	1.50	E1-Propanolol D7
E1-Tramadol	*0.7±0.01*	*0.25-200*	*0.997*	*0.05*	*0.25*	*MDMA D5*
E2-10,11-dihydro-10-hydroxycarbamazepine	1.1±0.01	1.5-800	0.998	0.50	1.50	Carbamazepine 13C6
E2-Alprenolol	0.3±0.01	0.25-500	0.998	0.05	0.25	E2-Attenolol D7
E2-Atenolol	1.0±0.002	0.75-600	0.999	0.25	0.75	E2-Atenolol D7
E2-Bisoprolol	1.0±0.01	0.75-400	1.000	0.25	0.75	E2-Metoprolol D7
E2-Metoprolol	1.0±0.01	2.5-200	0.999	1.00	2.5	E2-Metoprolol D7
E2-Mirtazapine	1.0±0.01	0.25-200	0.999	0.05	0.25	E2-Mirtazapine D3
E2-Oxazepam	1.0±0.004	0.5-400	0.998	0.01	0.50	E2-Oxazepam D5
E2-Propanolol	1.0±0.003	0.75-400	0.997	0.25	0.75	E2-Propanolol D7
Ethylparaben	1.0±0.03	5-600	0.998	1.00	5.00	Methylparaben 13C6
Fexofenadine	*8.5±0.03*	*25-1000*	*0.993*	*10.00*	*25.00*	*Ibuprofen D3*
Griseofulvin	0.8±0.01	1-400	0.997	0.50	1.00	Praziquantrel D11
Heroin	1.0±0.02	0.5-800	0.999	0.10	0.50	Heroin D9
HMA	2.7±0.01	1-600	0.997	0.50	1.00	MDMA D5
HMMA	*2.1±0.01*	*0.1-200*	*0.999*	*0.05*	*0.10*	*MDMA D5*
Hydrocodone	1.0±0.02	5-400	0.997	1.00	5.00	Hydrocodone D6
Imatinib	1.7±0.01	0.1-200	0.999	0.05	0.10	Morphine D3
Imidacloprid	3.8±0.01	1-200	0.997	0.50	1.00	Metazachlor D6
Indoprofen	1.7±0.004	0.5-600	0.999	0.10	0.50	Carbamazepine 13C6
Iopromide	*1.1±0.01*	*10-400*	*0.994*	*5.00*	*10.00*	*Gabapentin D4*

Ketamine	1.0±0.01	0.5-600	0.998	0.10	0.50	Ketamine D4
Ketoprofen	2.3±0.03	10-1000	0.997	5.00	10.00	Ibuprofen D3
MDA	1.0±0.03	0.5-600	0.999	0.10	0.50	MDA D5
MDMA	1.0±0.01	0.5-400	0.998	0.10	0.50	MDMA D5
MDPV	0.5±0.01	0.05-200	0.998	0.01	0.05	MDMA D5
Memantine	*0.8±0.01*	*0.5-400*	*0.998*	*0.10*	*0.50*	*Codeine D6*
Mephedrone	1.0±0.03	0.05-400	0.998	0.01	0.05	Mephedrone D3
Metazachlor	1.0±0.02	1.5-400	0.998	0.50	1.50	Metazachlor D6
Methadone	1.0±0.01	0.1-600	0.998	0.05	0.10	Methadone D9
Methamphetamine	1.0±0.01	0.05-800	0.999	0.01	0.05	Methamphetamine D5
Methylparaben	1.0±0.03	5-600	0.997	1.00	5.00	Methylparaben 13C6
Morphine	1.0±0.01	5-400	0.998	1.00	5.00	Morphine D3
Nordiazepam	1.0±0.01	0.5-200	0.999	0.10	0.50	Nordiazepam D5
Norephedrine	1.4±0.01	0.5-600	0.997	0.10	0.50	Flumequine 13C3
Normorphine	8.7±0.01	5-800	1.000	1.00	5.00	Metazachlor D6
Nortriptyline	1.0±0.01	5-400	0.998	1.00	5.00	Nortryptyline D3
O-Desmethylnaproxen	5.6±0.03	75-1000	0.991	25.00	75.00	Ibuprofen D3
Omeprazole	0.9±0.01	0.5-400	1.000	0.10	0.50	Quetiapine D8
Oxadiazon	0.2±0.03	1.5-400	0.998	0.50	1.50	E1-Oxazepam D5
Oxycodone	1.0±0.02	1.5-400	0.998	0.50	1.50	Oxycodone D6
Oxymorphone	*6.8±0.01*	*1.5-600*	*1.000*	*0.50*	*1.50*	*Metazachlor D6*
Pholcodine	0.6±0.01	5-200	0.998	1.00	5.00	Gabapentin D4
Praziquantrel	1.0±0.01	0.05-400	0.998	0.01	0.05	Praziquantrel D11
Propylparaben	1.0±0.03	5-800	0.999	1.00	5.00	Methylparaben 13C6
Quetiapine	1.0±0.02	0.1-400	0.998	0.05	0.10	Quetiapine D8
Risperidone	0.5±0.01	0.05-400	0.998	0.01	0.05	Gabapentin D4
Salbutamol	3.2±0.01	1-200	0.997	0.05	0.10	Diazepam D5
Sotalol	0.7±0.01	0.5-400	0.999	0.10	0.50	Gabapentin D4
Sulphadiazine	0.9±0.003	0.1-400	0.999	0.05	0.10	E2-Propanolol D7
Sulphamethoxazole	0.7±0.02	0.1-1000	0.998	0.05	0.10	Ofloxacin D3
Sulphapyridine	0.9±0.003	0.5-200	0.998	0.10	0.50	Benzoylecgonine D8
Terbutaline	1.0±0.02	25-800	0.997	10.00	25.00	Fluoxetine D5
Terbuthylazine	0.2±0.01	0.15-400	0.999	0.05	0.15	E1-Oxazepam D5
Tetramisole	1.0±0.01	1.5-600	0.999	0.50	1.50	Tetramisole D5
Thiamethoxam	*1.1±0.02*	*5-200*	*0.999*	*1.00*	*5.00*	*Quetiapine D8*
Triclosan	*1.1±0.04*	*15-1000*	*0.991*	*5.00*	*15.00*	*Ibuprofen D3*
Valsartan	14.2±0.03	15-1000	0.998	5.00	15.00	Ibuprofen D3
Vardenafil	0.9±0.01	0.05-400	0.998	0.01	0.05	Gabapentin D4
Zolpidem	0.9±0.01	0.5-600	0.999	0.10	0.50	Hydrocodone D6

Table S8. Average absolute recoveries (%) for all analytes analysed in the method, even if not developed further (n=3)

Analyte	River water			Wastewater		
	10 µg L^{-1}	50 µg L^{-1}	200 µg L^{-1}	10 µg L^{-1}	50 µg L^{-1}	200 µg L^{-1}
1,7 dimethylxantine	130.38	51.33	59.65	7320.58	2303.42	318.38
2-Hydroxyibuprofen	18.88	42.19	27.30	16890.07	1991.20	226.19
8-isoF2B	0.00	0.00	101.45	0.00	258.28	95.79
Acetaminophen	106.09	57.33	87.86	8642.40	1522.31	-118.34
Anhydroecgonine methylester	22.99	41.17	48.74	27.85	42.26	45.70
Aminorex	18.02	29.83	32.12	26.73	36.02	41.64
Amphetamine	30.76	59.35	60.77	22.22	28.40	32.16
Azathioprine	0.00	59.68	61.18	30.63	51.60	55.33
Benzophenone-1	29.48	67.94	59.97	75.64	113.44	85.44
Benzophenone-3	9.99	46.16	56.54	-187.79	33.62	54.26
Benzophenone-4	180.31	101.10	79.60	-201.52	145.17	85.40
Benzoylecgonine	50.58	83.03	82.57	47.53	67.41	52.26
Benzylpiperizine	9.12	11.89	23.56	17.58	23.59	32.26
Bezafibrate	57.66	69.82	66.44	67.27	95.59	77.57
Bicalutamide	110.59	72.76	80.32	68.74	13.20	36.45
Buprenorphine	27.70	39.47	56.19	7.11	34.39	40.00
Caffeine	53.67	63.29	85.61	349.13	-34.88	-33.04
Candesartan Cilexetil	20.02	22.92	24.91	4.29	22.82	20.79
Capecitabine	43.75	58.78	63.93	39.60	52.68	55.40
Carbamazepine	48.48	75.00	75.99	50.15	60.47	53.33
Carbamazepine 10,11 epoxide	51.92	75.83	75.37	44.51	65.34	60.60
Carprofen	23.20	68.68	53.29	32.20	61.04	55.09
Chlorpyrifos	-272.63	17.16	21.34	-127.30	16.89	16.63
Cimetidine	21.22	53.14	53.14	62.67	76.14	54.92
Citalopram	34.36	55.57	73.66	11.26	27.94	31.51
Clothiniadin	46.84	69.70	74.66	41.18	49.96	52.04
Cocaethylene	44.81	64.54	71.64	22.24	34.79	38.40
Cocaine	38.96	64.34	69.73	20.35	33.69	37.12
Codeine	31.17	67.54	90.62	166.17	56.83	48.69
Cotinine	26.76	56.98	78.36	34.53	22.55	26.09
Creatinine	-2.85	0.18	0.25	509.83	137.81	-0.38
Cytarabine	-1.58	-0.10	0.52	-1.35	1.73	0.42
Desmethylcitalopram	26.23	39.21	57.52	24.39	46.05	49.95
Desmethylvenlafaxine	23.23	70.99	81.25	73.41	62.60	49.78
Desvenlafaxine	29.49	71.30	79.91	68.06	63.47	47.64
DHMA	11.98	57.91	52.65	95.89	30.14	30.52
Diazepam	56.15	73.41	82.00	41.31	45.23	42.35
Diazinon	35.90	47.89	42.83	40.18	58.87	53.56
Diclofenac	49.80	77.11	67.47	53.64	90.57	75.64
Dihydrocodeine	52.79	70.88	85.94	47.77	56.35	57.27
Dihydroketoprofen	104.48	89.07	69.86	89.69	62.65	71.85
Dihydromorphine	60.70	69.61	75.13	63.09	53.68	48.70
Diltiazem	35.50	54.61	60.67	7.31	18.30	19.58
Duloxetine	9.58	14.48	22.21	0.83	25.53	27.02
E1-10,11-dihydro-10-hydroxycarbamazepine	53.52	79.18	90.76	34.85	59.09	65.89
E1-Alprenolol	48.53	49.23	70.17	19.40	35.84	38.73
E1-Atenolol	46.60	65.81	69.80	-9.66	59.69	53.21
E1-Bisoprolol	38.92	59.11	77.48	34.95	55.82	57.23
E1-Metoprolol	45.99	64.10	77.30	30.54	47.92	51.58
E1-Mirtazapine	42.94	70.68	85.65	17.04	30.42	35.68
E1-Oxazepam	55.45	68.32	67.12	51.25	54.68	51.09
E1-Propanolol	35.49	51.80	62.29	53.72	67.84	63.91
E1-Tramadol	32.06	73.71	78.75	3.51	21.80	25.21
E2-10,11-dihydro-10-hydroxycarbamazepine	50.83	79.73	80.88	47.71	66.05	62.26
E2-Alprenolol	47.04	49.31	64.76	43.13	51.43	48.38
E2-Atenolol	55.74	67.86	75.00	56.15	74.97	61.10
E2-Bisoprolol	40.08	58.74	68.24	55.38	81.21	71.12
E2-Metoprolol	39.85	58.00	69.15	65.86	75.87	69.59
E2-Mirtazapine	54.58	70.00	88.17	27.54	41.84	44.32
E2-Oxazepam	61.50	69.90	67.28	39.36	45.81	44.21
E2-Propanolol	37.05	51.71	65.77	27.51	51.65	53.63
E2-Tramadol	38.70	73.92	75.55	14.33	48.27	42.98
Ephedrine	6.70	8.08	17.89	-23.58	23.67	29.30
Ethylparaben	59.78	87.00	78.39	0.00	127.87	93.85
Fexofenadine	97.97	74.04	67.01	75.84	72.29	68.28
Flufenacet	32.58	46.04	56.01	24.01	31.12	33.15
Fluoxetine	10.94	17.39	29.63	1.70	11.01	12.81

Furosemide	3.53	1.85	1.12	4.84	13.23	5.93
Gabapentin	15.31	0.96	1.40	56.91	9.93	2.54
Gemfibrozil	448.09	68.28	69.93	-519.74	-33.21	15.32
Gliclazide	30.89	59.29	66.52	42.76	51.47	50.69
Griseofulvin	62.76	75.18	95.44	42.34	38.45	49.48
Heroin	28.87	41.02	44.01	14.62	18.22	19.55
HMA	19.75	37.29	44.48	13.54	35.33	36.28
HMMA	45.31	65.03	70.37	44.54	59.97	57.87
HNE-MA	0.00	98.95	64.79	0.00	66.50	79.25
Hydrocodone	42.33	68.62	90.08	85.42	56.98	59.50
Ibuprofen	0.00	116.96	102.88	6291.11	2364.50	562.20
Imatinib	42.45	50.63	55.69	35.65	55.29	49.51
Imazalil sulphate	22.87	48.64	65.00	2.88	12.00	16.60
Imidacloprid	50.06	79.46	87.27	61.58	70.02	66.97
Indoprofen	50.88	68.24	68.29	32.94	44.57	43.67
Iopromide	43.82	71.14	77.87	107.35	87.92	60.68
Irbesartan	47.04	66.20	65.94	44.94	57.07	51.62
Ketamine	49.23	73.70	74.37	28.93	35.63	34.75
Ketoprofen	30.29	87.24	79.39	0.00	83.54	82.07
MDA	22.63	47.14	49.70	29.07	44.36	57.03
MDMA	37.05	64.01	71.57	29.25	38.11	40.49
MDPV	40.48	54.45	68.77	25.76	37.23	37.18
Memantine	38.50	66.94	56.87	43.38	60.00	71.88
Mephedrone	16.15	33.76	41.91	8.52	15.42	21.19
Metazachlor	61.03	74.27	77.81	15.22	39.11	45.58
Methadone	52.35	59.04	68.91	13.90	34.98	36.81
Methamphetamine	20.61	52.81	40.76	14.68	19.52	24.49
Methylparaben	0.00	92.80	76.52	201.09	158.44	97.57
Morphine	49.04	73.39	81.15	150.38	94.67	68.89
N-desmethyl tramadol	19.62	31.91	45.39	37.13	53.31	48.49
N-Guanylurea	-20.70	-3.03	0.11	-6.66	0.04	1.00
Nicotine	-3.60	35.06	40.05	113.06	42.10	28.70
Norcodeine	22.85	21.93	57.17	47.32	30.54	41.45
Nordiazepam	57.15	73.94	81.20	47.52	59.81	56.70
Norephedrine	13.18	16.22	27.49	25.63	30.38	29.36
Norfluoxetine	4.15	7.81	11.92	4.02	13.61	18.90
Normorphine	2.57	31.17	52.07	36.88	51.19	43.43
Noroxycodone	9.93	6.56	17.04	14.64	23.32	26.07
Nortriptyline	21.02	26.26	33.56	13.42	36.69	38.99
O-6-MAM	48.89	83.27	117.30	30.24	55.98	64.73
O-desmethyl tramadol	42.29	73.16	79.00	28.73	48.39	45.60
O-Desmethylnaproxen	0.00	62.40	66.56	0.00	95.71	96.27
Omeprazole	72.68	86.54	75.22	80.08	73.43	54.70
Orlistat	5.60	7.72	11.04	1.73	1.41	1.46
Orlistat	5.18	7.29	10.79	-1.65	1.07	1.41
Oxadiazon	40.26	37.82	39.99	17.69	26.20	25.26
Oxycodone	64.42	74.69	94.18	43.24	41.02	50.38
Oxymorphone	0.00	6.28	17.37	15.14	23.61	28.13
Pholcodine	56.25	76.65	83.27	91.65	72.15	70.54
PMA	30.95	36.10	49.82	0.00	106.43	69.58
Praziquantrel	50.65	78.00	97.41	39.75	51.81	60.21
Pregabalin	0.00	0.00	0.00	7.79	2.51	0.96
Propylparaben	102.92	102.23	86.18	370.29	157.29	106.81
Quetiapine	53.54	67.78	76.65	47.64	55.11	51.89
Ranitidine	28.09	56.33	66.60	240.41	111.69	53.71
Risperidone	50.32	60.62	68.23	43.74	62.09	56.19
Salbutamol	49.45	70.32	76.68	15.78	35.59	37.86
Sertraline	16.33	21.90	31.69	9.63	17.69	17.43
Sitagliptin	-3.95	15.47	24.62	34.13	36.23	32.69
Sotalol	40.86	53.02	68.76	51.81	59.03	52.03
Sulfasalazine	-52.84	12.12	19.67	-13.91	60.34	55.25
Sulphadiazine	35.36	40.65	35.99	40.49	42.05	36.80
Sulphamethoxazole	40.36	59.42	66.30	31.32	51.83	49.21
Sulphapyridine	44.29	62.24	69.41	119.96	78.46	50.55
Terbutaline	12.92	13.09	6.78	10.59	9.46	3.46
Terbuthylazine	40.37	54.88	59.43	30.56	37.61	37.34
Tetramisole	48.29	64.11	74.83	28.05	33.49	33.62
Thiamethoxam	68.36	84.84	102.92	42.42	67.96	74.71
Triclosan	4.71	225.85	45.81	222.34	211.87	46.72
Valsartan	17.95	82.36	72.78	45.83	85.68	72.56
Vardenafil	52.77	67.45	69.07	44.94	71.44	70.32

Venlafaxine	49.69	69.54	73.31	11.59	29.03	29.10
Zolpidem	55.60	76.28	81.95	36.63	63.45	54.91

Table S9. Instrument accuracy and precision assessed over a period of one week (semiquantitative compounds are presented in italics))

Analyte	Accuracy (%)		Intraday precision (% RSD)		Interday precision (Average % RSD)	
	Average (n = 9)	SD	Average (n = 9)	SD	Average (n = 3)	SD
Aminorex	99	7.4	4	0.7	3	1.5
Anhydroecgonine methylester	101	3.8	2	1.3	2	0.9
Benzophenone-1	107	6.9	6	3.8	19	3.4
Benzophenone-4	106	26.0	7	1.1	14	7.7
Benzoylecgonine	102	5.2	1	1.0	1	0.6
Benzylpiperizine	*99*	*7.2*	*2*	*0.1*	*2*	*0.2*
Bezafibrate	123	11.6	7	3.0	16	0.2
Buprenorphine	100	8.8	5	1.3	6	1.5
Candesartan Cilexetil	*99*	*9.7*	*5*	*4.3*	*5*	*4.2*
Carbamazepine	101	5.9	2	1.0	2	0.6
Carbamazepine 10,11 epoxide	97	6.7	3	1.6	3	2.2
Carprofen	*98*	*0.3*	*6*	*2.1*	*15*	*1.6*
Citalopram	98	18.0	3	0.4	2	0.4
Clothiniadin	97	10.9	2	0.6	2	0.9
Cocaethylene	105	7.3	1	0.3	2	0.7
Cocaine	103	6.3	3	1.7	3	1.0
Codeine	99	13.6	4	0.1	5	1.2
Cotinine	99	6.9	3	1.2	2	0.2
Desmethylcitalopram	99	13.0	3	1.6	3	0.4
DHMA	*107*	*4.9*	*12*	*9.1*	*7*	*2.8*
Diazepam	98	3.2	2	1.2	2	0.7
Diazinon	103	4.8	5	3.0	5	2.5
Diclofenac	116	0.7	9	6.7	17	0.2
Dihydrocodeine	103	9.3	4	1.1	4	0.6
Dihydroketoprofen	112	18.3	5	0.8	15	0.4
Dihydromorphine	100	13.5	3	1.5	3	2.3
Diltiazem	*106*	*15.8*	*4*	*0.9*	*3*	*0.6*
Duloxetine	*106*	*15.7*	*4*	*1.6*	*4*	*1.6*
E1-10,11-dihydro-10-hydroxycarbamazepine	92	5.7	4	0.8	4	3.1
E1-Alprenolol	105	8.4	2	1.0	3	0.7
E1-Atenolol	96	6.4	1	0.8	2	0.8
E1-Bisoprolol	95	4.1	2	1.0	3	1.0
E1-Metoprolol	95	3.0	2	0.8	4	1.2
E1-Mirtazapine	93	3.4	2	0.0	2	0.3
E1-Oxazepam	100	9.1	3	0.8	4	0.3
E1-Propanolol	95	9.6	2	1.4	3	0.1
E1-Tramadol	*90*	*6.3*	*3*	*0.1*	*3*	*0.3*
E2-10,11-dihydro-10-hydroxycarbamazepine	95	5.0	3	0.5	2	0.4
E2-Alprenolol	109	5.9	2	1.2	2	0.8
E2-Atenolol	95	4.7	2	1.1	2	0.9
E2-Bisoprolol	94	5.7	2	0.6	2	0.5
E2-Metoprolol	99	12.4	2	0.1	3	1.8

E2-Mirtazapine	97	3.1	2	0.6	2	0.1
E2-Oxazepam	101	13.4	2	1.2	2	0.7
E2-Propanolol	91	3.5	3	0.6	3	0.1
Ethylparaben	103	8.6	8	0.7	11	3.9
Fexofenadine	*104*	*2.8*	*2*	*0.7*	*14*	*12.4*
Griseofulvin	97	7.1	4	0.7	4	0.8
Heroin	98	14.5	3	0.8	4	0.6
HMA	96	12.6	3	1.9	3	1.0
HMMA	*108*	*12.0*	*3*	*2.0*	*2*	*0.9*
Hydrocodone	93	18.1	5	1.9	3	0.8
Imatinib	105	5.8	3	0.6	3	0.5
Imidacloprid	96	10.4	2	1.5	2	0.8
Indoprofen	86	6.4	5	4.2	4	3.0
Iopromide	*98*	*17.2*	*4*	*2.4*	*5*	*1.4*
Ketamine	103	7.9	1	1.0	3	0.1
Ketoprofen	127	9.7	7	5.9	17	3.4
MDA	99	10.4	1	0.1	1	0.1
MDMA	101	8.5	1	0.6	1	0.4
MDPV	104	9.4	3	0.8	3	0.4
Memantine	*100*	*7.6*	*5*	*1.5*	*4*	*1.8*
Mephedrone	105	4.1	2	0.5	3	0.2
Metazachlor	95	11.6	5	1.9	4	2.0
Methadone	104	7.7	3	0.6	2	0.6
Methamphetamine	94	10.6	2	0.2	3	0.4
Methylparaben	99	8.3	6	0.7	9	5.4
Morphine	103	11.0	5	1.6	4	0.3
Nordiazepam	102	5.9	2	0.6	2	0.2
Norephedrine	102	17.8	7	6.4	3	2.4
Normorphine	93	16.6	5	3.4	5	1.8
Nortriptyline	97	12.5	3	1.8	4	0.5
O-Desmethylnaproxen	142	58.3	12	8.1	11	4.6
Omeprazole	106	8.9	1	1.4	2	0.4
Oxadiazon	119	11.6	6	2.2	6	2.3
Oxycodone	99	12.6	3	1.5	3	1.3
Oxymorphone	*96*	*8.8*	*3*	*2.2*	*3*	*1.1*
Pholcodine	100	9.3	4	3.9	4	2.1
Praziquantrel	101	5.7	3	0.4	3	0.5
Propylparaben	108	12.4	6	1.8	10	1.6
Quetiapine	101	15.9	1	0.3	2	0.7
Risperidone	102	15.2	2	0.7	2	0.5
Salbutamol	103	9.7	1	0.2	1	0.2
Sotalol	100	15.4	2	0.3	2	0.4
Sulphadiazine	96	4.6	5	1.3	4	0.2
Sulphamethoxazole	105	5.1	5	2.4	4	0.1
Sulphapyridine	98	5.2	3	1.2	2	1.0
Terbutaline	91	55.1	35	38.8	8	7.3
Terbuthylazine	106	10.7	5	3.7	4	0.9
Tetramisole	96	5.3	4	1.4	4	1.0
Thiamethoxam	*97*	*1.3*	*2*	*1.4*	*4*	*2.0*
Triclosan	*231*	*113*	*10*	*6.5*	*24*	*8.2*
Valsartan	105	7.8	8	2.3	15	0.7
Vardenafil	96	10.4	2	0.2	2	0.7
Zolpidem	104	6.3	8	5.8	5	3.5

Table S10. Average relative recoveries for all analytes analysed in the method, even if not developed further (n=3)

Analyte	River water			Wastewater		
	10 µg L^{-1}	50 µg L^{-1}	200 µg L^{-1}	10 µg L^{-1}	50 µg L^{-1}	200 µg L^{-1}

1,7 dimethylxantine	0.00	110.56	88.46	61862.73	7155.72	1111.20
2-Hydroxyibuprofen	10.39	53.75	32.63	4039.25	1099.68	159.46
8-isoF2B	0.00	0.00	145.67	0.00	234.65	111.65
Acetaminophen	0.00	39.34	81.64	-135882.32	-30190.06	-7853.75
Anhydroecgonine methylester	0.00	47.85	68.24	0.00	110.92	129.70
Aminorex	0.00	61.76	67.19	0.00	30.59	61.38
Amphetamine	0.00	90.65	102.36	0.00	108.83	106.64
Azathioprine	106.73	101.85	143.43	-1.19	-150.11	690.10
Benzophenone-1	0.00	51.02	69.57	128.18	90.18	87.55
Benzophenone-3	-31.17	-742.53	117.79	147.69	-543.44	218.70
Benzophenone-4	0.00	93.04	100.29	0.00	86.48	91.08
Benzoylecgonine	86.46	102.96	112.09	65.07	95.11	104.86
Benzylpiperizine	36.26	26.75	48.54	50.68	46.16	51.13
Bezafibrate	0.00	53.94	78.97	0.00	61.67	75.90
Bicalutamide	136.54	56.24	94.07	173.08	12.35	57.55
Buprenorphine	39.34	53.10	65.38	68.01	56.16	57.79
Caffeine	103.80	94.05	105.17	-4020.34	-1532.72	-125.69
Candesartan Cilexetil	72.91	40.60	27.88	89.90	72.68	49.45
Capecitabine	35.50	23.22	19.76	8.89	61.75	80.55
Carbamazepine	76.65	93.82	99.92	80.14	89.22	96.79
Carbamazepine 10,11 epoxide	68.68	92.43	98.12	64.14	99.44	107.35
Carprofen	0.00	63.14	61.33	49.19	36.74	56.48
Chlorpyrifos	0.00	22.56	16.56	-220.00	42.68	38.43
Cimetidine	102.77	122.56	57.50	2.43	-190.70	290.87
Citalopram	94.56	76.60	78.02	0.00	45.23	68.85
Clothiniadin	59.56	89.64	86.44	92.75	117.81	101.84
Cocaethylene	0.00	83.07	96.29	93.51	93.36	96.83
Cocaine	0.00	88.43	89.94	0.00	86.68	92.82
Codeine	160.55	96.79	97.11	0.00	52.91	84.02
Cotinine	123.95	102.48	99.31	0.00	65.66	89.11
Creatinine	0.82	0.41	0.09	-42.97	140.16	-3.23
Cytarabine	-333.81	-57.39	-10.08	-1336.61	-226.00	-43.16
Desmethylcitalopram	94.75	76.71	58.03	138.63	133.54	103.76
Desmethylvenlafaxine	111.68	109.38	77.72	-351.42	-139.77	65.46
Desvenlafaxine	124.62	111.71	80.87	-744.51	-203.87	69.80
DHMA	0.00	85.75	77.30	0.00	46.58	61.28
Diazepam	71.10	87.78	92.16	91.03	96.84	90.29
Diazinon	77.71	67.32	49.94	120.15	141.31	104.41
Diclofenac	0.00	84.73	79.95	87.30	54.06	82.71
Dihydrocodeine	87.90	82.89	88.82	0.00	77.22	83.97
Dihydroketoprofen	0.00	74.47	97.87	0.00	38.87	75.01
Dihydromorphine	91.75	93.84	85.74	111.26	85.26	68.65
Diltiazem	84.26	72.69	69.02	109.47	58.05	36.55
Duloxetine	27.17	19.07	26.29	55.86	46.38	44.05
E1-10,11-dihydro-10-hydroxycarbamazepine	0.00	96.78	125.36	0.00	98.52	125.92
E1-Alprenolol	0.00	69.32	96.88	0.00	60.72	70.67
E1-Atenolol	82.72	98.37	98.16	0.00	93.08	95.97
E1-Bisoprolol	77.17	83.75	101.15	65.40	92.01	109.38
E1-Metoprolol	72.74	88.67	100.40	61.93	81.10	97.81
E1-Mirtazapine	70.48	80.13	90.37	0.00	75.60	81.29
E1-Oxazepam	75.63	91.42	89.28	63.80	95.14	78.04
E1-Propanolol	79.59	89.97	96.46	72.60	86.76	96.99
E1-Tramadol	60.43	103.27	101.81	0.00	26.29	48.46
E2-10,11-dihydro-10-hydroxycarbamazepine	94.08	101.80	107.65	122.08	104.43	113.97
E2-Alprenolol	58.45	63.54	85.62	0.00	70.21	73.95
E2-Atenolol	75.10	89.05	98.99	0.00	96.51	94.27
E2-Bisoprolol	76.18	83.58	99.03	0.00	86.88	93.79
E2-Metoprolol	64.96	79.82	100.46	100.44	84.47	93.59
E2-Mirtazapine	66.29	83.89	90.74	0.00	80.17	80.51
E2-Oxazepam	95.12	100.06	87.86	69.27	91.51	75.21
E2-Propanolol	96.41	91.41	91.35	0.00	86.05	88.21
E2-Tramadol	80.74	107.43	110.59	0.00	31.50	54.53
Ephedrine	37.40	40.14	48.09	-100.62	36.96	60.39
Ethylparaben	78.00	89.67	106.35	0.00	82.98	91.94
Fexofenadine	0.00	97.51	80.65	0.00	34.79	64.93
Flufenacet	325.72	54.85	66.33	179.85	73.90	66.89
Fluoxetine	58.20	51.17	51.08	-54.15	29.26	40.06
Furosemide	132.16	116.42	0.00	133.15	114.80	0.00
Gabapentin	92.70	20.31	6.49	-14983.04	-3829.28	-851.19
Gemfibrozil	525.36	-467.06	53.79	1561.83	-1579.26	-116.71
Gliclazide	-2.29	-51.17	-2459.15	-3.65	-57.30	-2526.93

Griseofulvin	88.37	82.35	94.53	96.58	69.61	84.48
Heroin	84.28	86.55	93.54	0.00	89.56	84.95
HMA	66.00	53.80	55.72	80.22	78.37	71.47
HMMA	69.25	83.71	90.31	159.22	150.74	130.34
HNE-MA	0.00	37.60	100.26	0.00	79.46	150.77
Hydrocodone	103.31	88.79	89.88	0.00	65.79	81.97
Ibuprofen	0.00	138.19	149.28	0.00	699.53	130.70
Imatinib	76.30	63.24	64.91	125.66	96.07	79.54
Imazalil sulphate	0.00	70.40	93.45	0.00	30.49	47.19
Imidacloprid	103.26	108.38	101.51	120.74	160.62	129.84
Indoprofen	63.68	84.53	88.63	66.73	66.47	76.99
Iopromide	95.99	95.23	74.24	0.00	175.85	149.24
Irbesartan	0.00	47.86	72.17	21.78	42.36	49.64
Ketamine	68.73	95.64	110.65	93.57	109.17	108.44
Ketoprofen	0.00	64.57	122.99	0.00	48.64	86.97
MDA	62.42	83.74	64.83	108.12	82.64	75.40
MDMA	67.35	83.44	91.82	66.68	89.17	91.51
MDPV	66.38	70.30	88.26	83.99	92.22	83.60
Memantine	69.26	115.51	101.28	0.00	151.50	170.19
Mephedrone	0.00	61.23	90.98	0.00	53.90	70.42
Metazachlor	99.19	99.20	89.86	63.17	95.55	89.13
Methadone	0.00	69.36	88.92	0.00	73.43	83.11
Methamphetamine	0.00	79.09	79.98	96.86	84.54	87.58
Methylparaben	0.00	98.28	100.15	0.00	103.82	100.58
Morphine	99.32	100.70	93.59	0.00	108.61	94.87
N-desmethyl tramadol	46.87	64.34	97.27	95.26	263.88	215.37
N-Guanylurea	-95790.65	-1016.78	-230.66	-34802.26	-366.18	-81.29
Nicotine	-2.74	-21555.17	75.52	-178.01	-56282.76	146.11
Norcodeine	21.20	24.01	58.83	-31.70	43.53	60.32
Nordiazepam	75.55	83.52	93.57	101.73	94.51	92.24
Norephedrine	88.42	83.33	73.82	84.76	69.04	62.40
Norfluoxetine	7.81	21.26	20.54	-106.22	39.69	60.90
Normorphine	45.79	46.77	60.91	0.00	71.90	60.60
Noroxycodone	-12.32	5.09	16.85	-13.43	49.76	47.73
Nortriptyline	63.23	62.55	59.25	88.77	65.00	57.94
O-6-MAM	0.00	193.32	272.59	-686.70	379.84	320.14
O-desmethyl tramadol	110.24	148.92	170.38	2.61	235.49	203.46
O-Desmethylnaproxen	0.00	60.71	82.75	0.00	54.04	121.92
Omeprazole	100.52	105.24	78.25	0.00	145.58	119.15
Orlistat	0.00	0.57	12.48	-197.37	-7.50	0.00
Orlistat	17.62	13.13	13.56	0.00	7.65	5.33
Oxadiazon	87.36	60.95	46.89	96.81	75.82	50.67
Oxycodone	84.82	93.36	93.98	123.41	95.08	93.49
Oxymorphone	13.75	8.97	17.67	55.07	41.19	40.08
Pholcodine	93.94	89.51	85.71	121.07	103.92	100.87
PMA	270.74	191.36	139.39	0.00	225.09	150.46
Praziquantrel	72.68	85.39	96.94	90.38	93.18	103.15
Pregabalin	0.00	0.00	0.00	-11.51	-4.66	0.58
Propylparaben	24.53	86.55	108.29	56.37	100.95	106.58
Quetiapine	77.32	79.95	88.81	138.45	95.59	83.75
Ranitidine	0.00	117.09	118.99	-249112.12	-38521.13	-7552.42
Risperidone	87.66	76.86	77.12	96.91	97.51	85.67
Salbutamol	99.98	95.80	77.40	0.00	85.99	102.89
Sertraline	14.52	36.32	46.60	-218.14	-15.59	22.58
Sitagliptin	57.64	28.14	30.13	214.43	87.00	57.79
Sotalol	90.97	74.04	69.74	0.00	149.67	142.30
Sulfasalazine	0.00	0.00	20.86	0.00	39.54	49.62
Sulphadiazine	53.63	66.77	49.83	73.20	74.72	62.25
Sulphamethoxazole	114.69	104.15	138.23	0.00	120.45	116.93
Sulphapyridine	87.66	78.41	95.06	0.00	121.17	101.46
Terbutaline	98.55	98.93	0.00	82.53	85.55	0.00
Terbuthylazine	81.22	75.56	68.91	94.24	92.72	73.26
Tetramisole	0.00	89.42	103.01	0.00	93.17	92.79
Thiamethoxam	105.91	112.66	121.21	130.31	168.11	149.41
Triclosan	0.00	129.08	51.90	0.00	40.80	30.57
Valsartan	0.00	75.95	84.27	69.78	75.52	78.20
Vardenafil	88.49	83.54	78.09	97.10	107.83	105.12
Venlafaxine	124.94	97.53	75.43	-1022.94	-43.87	67.67
Zolpidem	77.51	91.88	91.53	85.35	135.28	116.15

This declaration concerns the article entitled:			
A new paradigm in public health assessment: Water fingerprinting for protein markers of public health using mass spectrometry			
Publication status (tick one) **Draft manuscript** [ ] **Submitted** [ ] **In review** [ ] **Accepted** [ ] **Published** [X]			
Publication details (reference)	DOI: 10.1016/j.trac.2019.115621 Rice, J., Kasprzyk-Hordern, B., 2019. A new paradigm in public health assessment: Water fingerprinting for protein markers of public health using mass spectrometry, *Trends in Analytical Chemistry*, 119		
Copyright status (tick the appropriate statement) I hold the copyright for this material [ ] Copyright is retained by the publisher, but I have been given permission to replicate the material here [X]			
Candidate's contribution to the paper (provide details, and also indicate as a percentage)	The candidate contributed to / considerably contributed to / predominantly executed the… Formulation of ideas: Candidate considerably contributed to the formulation of ideas in collaboration with Barbara Kasprzyk-Hordern Design of methodology: Candidate predominantly executed the design of methodology proposed in the paper in collaboration with Barbara Kasprzyk-Hordern Experimental work: N/A – literature review paper Presentation of data in journal format: Candidate predominantly executed the writing and formatting of the publication with assistance from Barbara Kasprzyk-Hordern		
Statement from Candidate	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature.		
Signed		**Date**	02/06/2020

Chapter four introduction

Chapter four marks a change in direction for the thesis by being both a literature review and a discussion of a new potential source of public health biomarkers. In this manner chapter four directly explores the third aim of the thesis “the exploration of proteins as potential new biomarkers of public health”, although it does include an in-depth discussion of current WBE biomarkers.

The narrative of the chapter begins by discussing how public health is currently measured, from clinical assessments that are used to build up a picture of public health from the assessment of individuals, to techniques like WBE that look at wastewater as equivalent to a pooled, population-wide urine sample. The narrative then merges the two concepts to suggest that proteins should be considered as biomarkers of public health.

The discussion throughout the chapter is focussed on urinary biomarkers of individual health as an under developed area of interest, with most FDA qualified biomarkers being found or analysed only in blood, either plasma or serum. In table 1, designed to show the breadth of analytes available from small molecules to proteins to genes, only KIM-1, albumin and cystatin C are FDA qualified biomarkers and are all biomarkers of kidney or renal injury in rats or humans, excluding albumin, and are all analysed via immunoassays. The chapter then discusses current WBE practise and how widely wastewater analysis has been used, and the range of biomarkers that have been identified. The possibility of linking urinary proteomics and WBE is then discussed, highlighting their shared usage of LC-MS/MS for biomarker analysis. In order to facilitate this, a range of selection criteria for urinary protein biomarkers is then outlined before presenting an initial series of biomarker candidates, including their urinary concentrations in healthy individuals.

An addendum of additional considerations and a more in-depth discussion of protein analysis, which was not appropriate for inclusion in the publication, is included at the end of the chapter on page 232.

A new paradigm in public health assessment: water fingerprinting for protein markers of public health using mass spectrometry

Jack Rice[1] and Barbara Kasprzyk-Hordern[1*]

[1] Department of Chemistry, University of Bath, Bath BA2 7AY, United Kingdom

*Corresponding author: Barbara Kasprzyk-Hordern, Department of Chemistry, University of Bath, Bath BA2 7AY, United Kingdom, Tel: +44(0)7971860993, e.mail: b.kasprzyk-hordern@bath.ac.uk

Abstract

Public health monitoring cannot currently provide real time and comprehensive information about the health of a community as it depends on individual data collection (e.g. surveys) and is limited by the cost of biomonitoring campaigns. To allow for better monitoring of public health the burden of accuracy must move from the participant, whose accuracy cannot always be determined, to an assay or model whose accuracy can be determined either mathematically or scientifically. Additionally, new techniques should reduce overall costs in data collection and collation, whilst also expanding the number of individuals included in a study. Water fingerprinting that originated from wastewater based epidemiology (WBE) has a strong potential to revolutionise public health monitoring. WBE is a tool used for the analysis of drugs of abuse in populations worldwide, and future developments in this field are currently focussed on an expanding range of molecular biomarkers such as pharmaceuticals. However there also exists the possibility to adapt this approach for the analysis of proteins, which are currently clinically limited to the analysis of individual patients. By combing the techniques of WBE and clinical proteomics there exists the possibility for near-real time, population wide, human biomonitoring of disease. This manuscript details the considerations and stepping stones needed to allow for water proteome fingerprinting, as well as giving an overview of WBE and its applications; including the range of biomarkers, methods and populations currently examined.

Keywords: water fingerprinting, wastewater-based epidemiology, public health, protein markers

Introduction - Public health monitoring and its limitations

Public health monitoring cannot currently provide real time and comprehensive information about the health of a community, limiting its ability for prediction and tracking of health events resulting from seasonal infections, environmental contamination (e.g. respiratory diseases resulting from air pollution), or long term monitoring of public health. This limitation is brought about by a reliance of current public health studies, such as NHS England's health survey for England [1], using methodologies based on individual data collection. These sources of data rely on practitioners and participants to have accurate knowledge of their health to collect accurate information, or rely on collecting biological samples from every participant. This limits their ability to provide information in real time as each individual piece of patient data must be collected and collated before any conclusions can be drawn. To allow for better monitoring of public health the burden of accuracy must move from the participant, whose accuracy cannot always be determined, to an assay or model whose accuracy can be determined either mathematically or scientifically. Additionally, new techniques should reduce overall costs in data collection and collation, whilst also expanding the number of individuals included in a study.

Molecular epidemiology in public health assessment

Molecular epidemiology provides a better tool for understanding public health by targeting specific health biomarkers. The burden of accuracy can then be moved from the individual to the methodology, whose accuracy can be verified. Clinical biomarkers range from (i) non-molecular markers such as blood pressure as a biomarker of heart disease, (ii) genetic biomarkers, (iii) small molecule metabolic biomarkers, and (iv) large molecules like proteins. The range of possible urinary biomarkers is summarised in Table 1 and highlights that urine can also be a source of biomarkers for non-urogenital diseases [2].

Table 1. Examples of urinary biomarkers.

Urinary marker Name	Class	Disease	Ref
Psoriasin	Protein	Bladder cancer	[2]
Telomerase	Protein		[3]
Nucleosides	Small molecule	Colorectal cancer	[4]
PCA3	Gene	Prostate cancer	[5]
GSTP1, RASSF2, HIST1H4K, TFAP2E, 9P21	Gene		[6]
Annexin 3, NMP22	Protein		[7]
Lipocalin 2	Protein	Breast cancer	[8]
1,4-dihydroxynonane mercapturic acid, 8-hydroxy-2-deoxguanosine, 8-isoprostane-F2alpha;	Small molecule	Oxidative stress	[9-11]
Porphobilinogen, 5-aminolevulinic acid	Small molecule	Acute intermittent porphyria	[12]
Adiponectin	Protein	Systemic lupus erythematosus	[13]
Pyridinolines	Small molecule	Osteoporosis	[14]
NGAL, KIM-1, IL-18, cystatin C, a1-microglobulin, fetuin-A, Gro-alpha, meprin	Protein	Acute Kidney Injury	[15]
Immunoglobulin G, transferrin, ceruloplasmin, albumin	Protein	Diabetic neuropathy	[16]
Nitric oxide	Small molecule	Acute respiratory distress syndrome	[17]
VEGF, PlGF, sFlt-1	Protein	Pre-eclampsia	[18]
AD7C-NTP	Protein	Alzheimer's disease	[19]

Clinical proteomics for individual health

Despite the wide range of biomarkers listed in Table 1 there is a perceived bottleneck in confirming the validity of protein biomarkers, which is broadly attributed to the time and expense required to take these biomarkers through clinical trials [20-22]. When compared to the cost of researching potential new biomarkers this leads to a range of potential biomarkers being identified, but very few of them being confirmed or used clinically. A proposed solution to this bottleneck is the introduction of liquid chromatography coupled with mass spectrometry techniques (LC-MS) for protein analysis as opposed to antibody based methods such as ELISA [22; 23]. The advantages of mass spectrometry over ELISA are that it enables greater multiplexing of samples at a lower cost per sample, despite an initial larger capital investment in equipment. Other advantages include the ability to multiplex analysis, either by analysing multiple biomarkers in a single run [24] or multiple different samples in a single run through isotopic labelling [25; 26].

Protein biomarkers are particularly ubiquitous within oncology, as the majority of FDA approved protein biomarkers are biomarkers of cancer [27]. Despite a current bias for blood serum or plasma biomarkers, only 2 of the FDA approved biomarkers are found in urine,

there is a growing interest in urinary biomarkers for clinical analysis as it can be more easily collected in large quantities and more willing given for repeat analysis, meaning an individual's health can more easily be tracked over time, such as when tracking disease progression [28]. As an example prostate specific antigen (PSA) is an FDA recognised biomarker of prostate cancer [27], which is analysed every 6-12 months in serum from men at risk of developing or with early stage prostate cancer, a process known as watchful waiting, [29; 30] to identify when intervention or treatment is necessary. Analysis of PSA in urine would potentially allow for this process to be carried out more frequently, or for a panel of biomarkers to be used for more accurate characterisation of disease state [31].

However the current clinical biomarker approach is still limited to the collection of data on individual health through analysis of blood or urine. Analysis of individual health can simply be scaled up for public health analysis but this presents its own challenges due to the large number of samples that would require collection and analysis [1; 32]. The largest of these studies [32] took place on a city-wide scale collected and analysed roughly 58,000 urine and 8600 blood samples, including controls, whilst also relying on questionnaires to screen participants into or out of the study. This approach allowed researchers [32] to collect data on the urinary concentration of one protein (C-reactive protein). Positive association between elevated levels of C-reactive protein and cardiovascular and renal risk factors was observed. However with this approach, only 10% of a population or approximately 8600 individuals were included in the study. New approaches are therefore urgently needed to provide more comprehensive datasets covering whole population and accounting for spatial and temporal variability.

Urban water fingerprinting for public health assessment: **the role of mass spectrometry in water fingerprinting for small molecules.**

Molecular epidemiology can be expanded to provide information on the health of a community through the use of urban water fingerprinting (UWF), where wastewater influent acts as a surrogate pooled urine sample for the entire population; meaning one representative sample can be collected for an entire population (Figure 1). For the study above [32] the city surveyed was served by one WWTP, meaning the whole population could have been examined using only one composite sample.

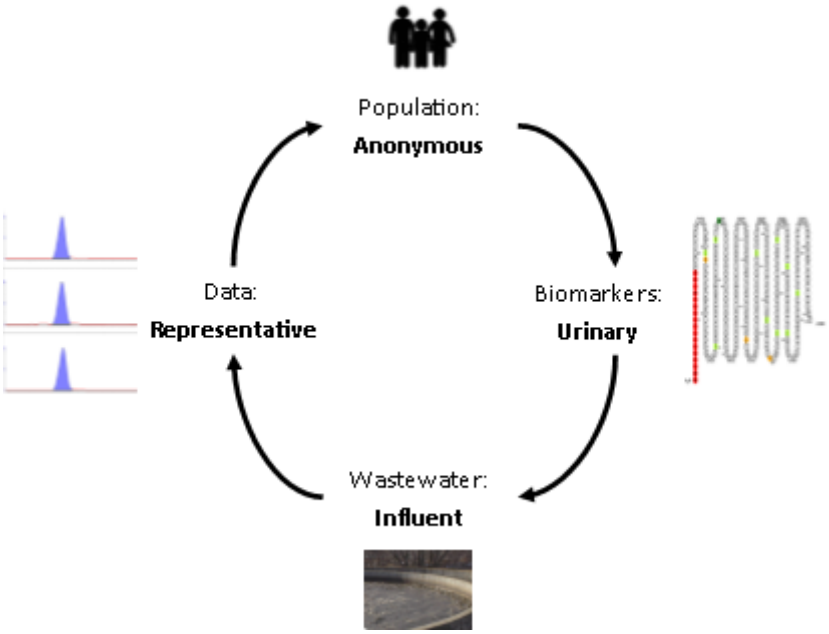


Figure 1. Water fingerprinting for public health assessment (note: the image of the protein: KLK3_HUMAN (PSA) [33]

This cutting-edge approach of extracting epidemiological information from urban water is also known as Wastewater-Based Epidemiology (WBE). The concept was introduced by [34] and followed by researchers worldwide [35-39]. In Europe WBE was developed in a strong transdisciplinary collaborative ethos within SCORE (www.score-cost.eu) and SEWPROF teams (www.sewprofitn.eu). WBE is currently used to report on world-wide illicit drug use trends [39] and feeds into the Europe-wide evidence based EWS managed by the European Agency for Drugs & Drug Addiction (EMCDDA, www.emcdda.europa.eu). In practice WBE can be divided into three stages: sample collection, sample analysis and back calculation, which are explained in detail in a guide published by the EMCDDA [40]. Sample collection typically involves the collection of time or flow proportional composite samples of influent wastewater from a wastewater treatment plant, over a 24h period. The composite sample is then transported back to the laboratory for analysis. Sample preparation often includes a sample concentration step, typically solid-phase extraction, before analysis using chromatography coupled to mass spectrometry to determine the analyte concentration. This measured concentration represents the amount of analyte in the sample and cannot be directly compared to other samples and needs normalisation. The back calculation step takes this measured concentration and normalises it using parameters such as flow and population size to generate a value that can be compared between treatment works and beyond to cities and countries.

Currently WBE is applied to the analysis of small molecules, such as pharmaceuticals, drugs of abuse and their metabolites [41]. Emerging analysis focuses on the importance of stereoselective human metabolism [42; 43], or endogenous chemicals formed in humans, i.e. biomarkers of oxidative stress [44], or on estimating the population contributing to the wastewater [36; 41; 45-47], or in matching wastewater and prescription or consumption data [48-51]. Powerful European sampling campaigns proved WBE's potential for comprehensive spatial and temporal community-wide (i) drug use assessment [52-59], (ii) pharmaceuticals and population biomarkers [48; 60], (iii) alcohol and tobacco use [61; 62], (iv) caffeine [63], (iii) pesticide and EDCs' exposure [64-66], (iv) air pollution and asthma [67] and (v) levels of oxidative stress [48]. The list of available biomarkers is presented below (Table 2).

Table. 2 Available biomarkers and studies undertaken so far.

Class	**Biomarkers**	**Population tested** (contributing to the sample)	**Comments**	**Ref**
Illicit drugs:				
Cocaine	Benzoylecgonine, Cocaine, Cocaethylene	111,037,591	• HMMA and HMA are metabolites of MDMA • O-6-MAM is a specific metabolite of Heroin • BEG is the major metabolite of cocaine and the BEG/Cocaine ratio is used for back calculation. Cocaethylene is a specific metabolite of cocaine and alcohol co-consumption	[36-38; 42; 45; 51; 53-59; 61; 68-79]
MDMA	MDMA, HMMA, HMA			
MDEA	MDEA			
Cannabis	THC-COOH			
Amphetamine	Amphetamine			
Methamphetamine	Methamphetamine, Amphetamine			
Heroin	Heroin, O-6-MAM			
Ketamine	Ketamine, Norketamine			
LSD	OH-LSD			
Cathinone	Cathinone, Cathine, Methcathinone, Flephedrone			
Lifestyle chemicals:				
Alcohol (ethanol)	Ethyl sulphate	20,129,696		[48; 49; 61; 80; 81]
Tobacco (nicotine)	Cotinine, Menthol, Nitrosamines, Hydroxycotinine, Anatabine, Anabasine	15,162,427		[42; 47-49; 62; 78; 82-84]

Caffeine	Caffeine, 1-methylxanthine, 7-methylxanthine, 1,7-dimethylxanthine,1-methyluric acid, 1,7-dimethyluric acid	43,834,325		[36; 42; 46; 48; 60; 78]
Sweetener	Acesulphame	33,280,000		[36]
New psychoactive substances (NPS):				
Synthetic cathinones	MDPV, Methylone, Mephedrone, α-PVP, Ethylone, Butylone, Naphyrone	30,326,489	JWH-XYZ is a generic placeholder for over 450 synthetic cannabinoids identified by Clemens University. Examples include the cannabinoid JWH-018, better known as "Spice"[85].	[42; 75; 79; 86-88]
Piperazines	Benzylpiperazine, TFMPP, mCPP			
Amphetamine-like	PMA, MDA, PMMA, MPA, 4-MEC, 4-EMC, 4-Fluoroamphetamine			
N-Bombs (25-X-NBOMe)	25-I-NBOMe, 25-C-NBOMe, 25-B-NBOMe			
Synthetic cannabinoids	JWH-XYZ, AH-7921			
Hallucinogenic	Methoxetamine, 2C-I, 2C-T-2, 2C-T-7			
Pesticides:				
Triazines	Atrazine,	4,973,945		[64]
Pyrethroids	3-PBA			
Organophoshates	DEP, DETP, DMP, DMTP, Chlorphyrifos,			
Pharmaceuticals:				
Antihypertensive	Atenolol, Bisoprolol, Metoprolol, Losartan, Propanolol, Valsartan, Telmisartan, Hydrochlorthiazide	54,433,111	• EDDP is a metabolite of Methadone • Oxazepam, Temazepam and Nordiazepam are analysed as both pharmaceuticals and metabolites of Diazepam • Salicylic acid is analysed as a metabolite of Aspirin	[35; 36; 42; 48; 50; 73; 77-79; 89]
Opioids	Methadone, EDDP, Morphine, Codeine, Fentanyl, Tramadol, Buprenorphine, 3-β-D-morphine glucuronide			
Non-steroidal anti-inflammatory (NSAIDs)	Diclofenac, Paracetamol, Ibuprofen, Naproxen, Salicylic acid			
Ephedrines	Ephedrine, Pseudoephedrine			
Benzodiazepines	Diazepam, Oxazepam, Nitrazepam, Nordiazepam, Temazepam, Clonazepam,			

	Lorazepam, Alprazolam, Flunitrazepam, Etizolam, Phenazepam			
Anti-epileptic	Carbamazepine, Gabapentin			
Anti-depressant	Citalopram, Fluoxetine, Venlafaxine			
Anti-ADHD	Methylphenidate, Atomoxetine			
Contrast agent	Iopromide			
Anti-diabetic	Metformin			
Fluoroquinolones	Norfloxacin, Ofloxacin,	12,224,589	• Trimethoprim is used in conjunction with sulphonamides	[90]
Sulphonamides	Sulphamethoxazole			
Macrolides	Roxithromycin, Erythromycin Trimethoprim			
Short acting β-agonists	Salbutamol	2,300,000		[67]
Public health:				
Biomarkers of oxidative stress	8-isoPGF2Alpha	5,274,075	. All prostaglandins are potential biomarkers of oxidative stress	[48; 91]
	1,4-methylimidazoleacetic acid	2,761,423		[92]

The advantages of wastewater analysis are that the sample collected is more representative of the population (as the whole population continuously contributes to its wastewater in real time), is collected non-invasively, has low collection costs and is effectively anonymous due to contribution of material from many individuals. Participant anonymity is an important ethical consideration, and an advantage of WBE is that data gets easier to anonymise as larger populations are investigated. However, limitations include WBE's inability to identify the demographics of the population, meaning researchers would need to rely on censuses to collect data about the population contributing to the wastewater (Table 3).

Table 3. Advantages and limitations of WBE

Advantages	**Disadvantages**
Representative samples	No demographic information
Non-invasive sample collection	Specialised equipment required
Low sample collection costs	High-skilled staff required
Contributor anonymity	Ethically unsuitable for small populations
Applicable to large populations	Relatively small pool of biomarkers available

Urinary proteins as public health biomarkers: the role of mass spectrometry in water fingerprinting for proteins.

By coupling the individual focussed approaches of clinical proteomics with the non-invasive and representative sample collecting of WBE there exists the potential to achieve population wide proteomic monitoring. Whilst the analysis of proteins within wastewater has, to the authors' knowledge, never been attempted before it can share a lot of the same techniques and advances as other molecular epidemiological approaches. First amongst these is the use of mass spectrometry (MS) as a tool for screening for biomarkers followed by quantitative analysis [93], allowing for the development of robust methods whose accuracy and precision can be validated. MS is already used within proteomics both for the analysis of intact proteins (top-down) [94] and in the analysis of peptides (bottom-up) [95]. The use of bottom-up proteomics is of greater interest as the same range of spectrometers can be used in both WBE and peptide analysis.

Bottom-up LC-MS/MS analysis is increasingly used within clinical proteomics as a means of increasing sample through-put, reducing analysis cost per samples and decreasing manual sample preparation as compared to traditional immunoassays. The population study discussed earlier analysed C-reactive protein (CRP) using nephelometry [32], a light scattering technique from the formation of antibody-antigen particles in solution, whilst several papers published at the same period measure CRP in serum using LC-MS/MS. The use of nephelometry only allowed quantification down to 0.18mg/L of intact CRP, whilst MS based approaches were able to achieve quantify CRP between 1-1000 fmol/µL or 25-25000µg/L in a more complex matrix [96]. MS based approaches have been developed that achieve similar sensitivity to that of ELISA [97; 98] and avoid the issue of non-specific antibody binding [99] that could result from using as complex a matrix as wastewater. The use of isotopically labelled internal standards is common in MS-based analysis for both small molecules and proteins, with a range of protein quantifications strategies available including labelled peptides (AQUA) [23; 96; 100], labelled proteins (PSAQ) [101] and concatomers formed by linking several peptides of interest together like a synthetic protein (QConCat) [102]. Label free methods that do not use isotopically labelled standards are also available [103; 104], instead adding an exogenous protein such as adding bovine serum albumin to samples of lupin seeds[104]. Label free methods are likely unsuitable for public health purposes due to the complexity and highly variable nature of wastewater, as without a more thorough

understanding of the wastewater proteome it is impossible to say that any protein added as a standard would not be naturally present in wastewater.

Taken together current proteomic analysis techniques could be adapted for analysis of wastewater but where new ground must be broken is in the selection of biomarker targets. The following five criteria are suggested to be important for biomarker selection: (1) Urinary excretion – Wastewater analysis requires that the biomarker be excreted in urine; (2) Known biomarker-disease relationship – Specifically how excretion and wastewater concentrations change with disease state and progression; (3) Disease specificity – Important for targeted disease monitoring, although more general biomarkers should also be considered; (4) Biomarker stability – The biomarker must be stable both in-vivo and after excretion and sample collection and (5) High urinary concentration – To allow for quantification in wastewater

The use of non-specific biomarkers, such as biomarkers of inflammation like C-reactive protein [32] and interleukins [28], can potentially be used to monitor the overall health of a population. This can allow for better targeting of healthcare services and interventions to specific communities, whilst also allowing for the long term monitoring of community health across different populations. Further work is needed to assess biomarker stability in wastewater, but their storage within the bladder, for a period of hours, prior to excretion suggests good stability [21]. Using the criteria above the following biomarkers are suggested as being initial targets of interest (Table 4): prostate specific antigen (PSA), C-reactive protein (CRP), interleuekin-6 (IL-6), interleukin-8 (IL-8), podocin (PDC), anterior gradient protein 2 (AGR2) and uromodulin (URM). They are all excreted in urine and have been targeted in clinical studies that focussed on the relationship between a single biomarker and disease. All the biomarkers have a known biomarker disease relationship in urine, with the presence of disease generally increasing the biomarker concentration, although concentrations of URM have been observed to increase or decrease depending on the specific kidney disease [105].

Table 4. Proteins as potential biomarkers in WBE

Biomarker	**Function**	**Healthy urinary concentration**	**Reference**
Prostate specific antigen (PSA)	Prostate cancer Bladder cancer	Men: 11.24ng/mL (average age 30) Women (not using contraception): 1-35 pg/mL (Average age 30-31) Women (using contraception): 521 pg/mL	[21],[106; 107] [108]
C-reactive protein (CRP)	Inflammation Cardiovascular disease	Average:1.2µg/mL, Interquartile range: 0.54-2.76µg/mL	[32]
Interleukin-6 (IL-6)	Inflammation Urinary tract infections	1.6-5.28 pg/mL	[28; 109]
Interleukin-8 (IL-8)		7-12 pg/mL	[110]
Podocin (PDC)	Acute kidney injury Pre-eclampsia	4.05 ng/mL	[111] [112]
Anterior gradient protein 2 (AGR2)	Prostate cancer (as a ratio with PSA)	223.4pg/100µg of urinary protein AGR2/PSA ratio: 0.0045	[97]
Uromodulin (URM)	Chronic Kidney Disease (CKD) Hypertension, CVD	21-1344 ng/mL	[105; 113]

Prostate specific antigen is a well-studied biomarker for prostate cancer diagnosis and is used to complement more invasive methods of diagnosis [30; 114], including biopsies, as well as for monitoring changes in prostate health, known as watchful waiting. The analysis of urinary CRP for measuring renal function in a healthy population has already been discussed [32], but urinary-CRP is not as well studied as a serum/plasma CRP, which is used as a biomarker for cardiovascular disease, rheumatoid arthritis and other inflammation related diseases [96; 115; 116]. Similarly IL-6 and IL-8 are also biomarkers of inflammation and have been individually quantified in urine as biomarkers of urinary tract infections [28; 109; 110; 117; 118] as well as potential biomarkers of Lupus nephritis [119]. IL-8 has also been examined as a biomarker of diabetic nephropathy [120]. Podocin is a protein that originates from podocyte cells in the kidneys, which act like a molecular sieve for proteins, and has been examined as a biomarker of both kidney health [111] and for determination of pre-eclampsia [112]. Uromodulin is the most abundant protein in urine and is touted as a biomarker for accessing renal health [113], CKD and hypertension [105]. Uromodulin is also interesting as its expression can be both up and down regulated by disease [105], with uromodulin-associated kidney disease reducing the excreted concentration, whilst individuals with greater risk of CKD, hypertension or CVD could produce more uromodulin than those with a lower risk.

Quantification of expected protein levels in wastewater might prove challenging but not impossible. Protein levels vary from ng to mg L^{-1} in urine (Table 4). Assuming 100 times dilution of urine in wastewater, one should expect estimated levels to range between pg and µg L^{-1}. Whilst these very low concentrations seemed daunting, current proteomic methodologies were able to quantify proteins within the upper portion of this range. A recent publication [111] was able to quantify podocin at 0.5 µg/L by making use of solid phase extraction (SPE), which is also used within WBE, to increase the podocin concentration and remove unwanted interferences from the urinary matrix. For analysis in wastewater it is not unusual to use the same SPE methodology to concentrate samples up to 200 times [42; 121], meaning quantification in the low ng/L range would theoretically be possible by using current proteomic methods to analyse wastewater. More research is therefore needed to determine the best approaches for the detection and quantification of proteins in wastewater. Antibody based method have been used to help separate proteins in complex matrices, like serum and plasma, and lead to an increase sensitivity [99; 122; 123], such as a 1000x fold increase in the quantification of PSA after depletion of more abundant proteins in serum [122]. However, and as previously discussed, antibody based methods are designed to assist in the analysis of only one or two proteins at a time, whilst the strength of WBE lies in its ability to analyse potentially hundreds of different compounds at a time.

The combination of proteomics and WBE has the potential to allow for long term monitoring of public health, as well as identification of disease hot spots or the prediction of epidemics. Advances in the related field of WBE can easily be incorporated for the analysis of proteins, giving rise to the possibility of monitoring both overall public health and specific health concerns in near real time, as well as to verify the effectiveness of interventions or government policy. The differences in the broad physical and chemical properties of proteins, peptides and small molecules should be exploited to aid in their analysis. One such example is the presence of carboxylic acid and amine containing side groups within peptides can allow for their selective enrichment using ionic SPE stationary phases or separation using ion chromatography or HILIC [111; 122; 124], both of which can be readily adapted for use with wastewater matrices. The larger mass and size of proteins and peptides compared to small molecules also makes the use of techniques like size-exclusion chromatography and ion mobility mass spectrometry promising, but this requires a shift away from the techniques already used within WBE analysis [40] and those proposed for clinical proteomics [22; 23],

namely reverse-phase liquid chromatography and either triple quadrupole, quadrupole-time of flight or orbitrap mass spectrometers.

Further development of WBE will be driven by advances in the analytical approaches utilised, both from the point of selective and sensitive measurement of known markers, as well as the discovery of new biomarkers. Breakthrough in protein analysis is yet to be realised by advancements in high resolution mass spectrometry utilising both Orbitrap and TOF technology, which enable both sensitive and selective quantitative analysis as well as retrospective data mining.

Conclusions

Clinical proteomics is currently limited to analysis of individual health, with attempts being made to expand this to cover a population. In this way proteins are comparable to illicit drug and other smaller molecule analysis prior to the introduction of WBE. Clinical proteomics already shares many of WBE's methods of analysis, namely liquid chromatography and mass spectrometry, with WBE providing many advantages for population proteomics, including contributor anonymity and easier applicability to analysing large populations. As wastewater analysis advances to encompass a broader range of health biomarkers new developments can be applied to the analysis of proteins, which will allow for the monitoring of public health using an entirely new class of health biomarkers.

Acknowledgments

The support of the Leverhulme Trust (Project No RPG-2013-297) and Engineering and Physical Sciences Research Council (Project No EP/P028403/1) is greatly appreciated.

References

[1] NatCen social research, U.C.L., 2016. *Health Survey for England 2015* [Online]. ed. NHS Digital. Available from: [Accessed 22/09/2017].
[2] Jain, K.K., 2010. Handbook of Biomarkers. *Handbook of Biomarkers*, pp. 1-492.
[3] Weikert, S., Krause, H., Wolff, I., Christoph, F., Schrader, M., Emrich, T., Miller, K. and Muller, M., 2005. Quantitative evaluation of telomerase subunits in urine as biomarkers for noninvasive detection of bladder cancer. *International Journal of Cancer,* 117(2), pp. 274-280.
[4] Feng, B., Yue, F. and Zheng, M.H., 2009. URINARY MARKERS IN COLORECTAL CANCER. *Advances in Clinical Chemistry, Vol 47,* 47, pp. 45-57.
[5] Marks, L.S., Fradet, Y., Deras, I.L., Blase, A., Mathis, J., Aubin, S.M.J., Cancio, A.T., Desaulniers, M., Ellis, W.J., Rittenhouse, H. and Groskopf, J., 2007. PCA3 molecular urine assay for prostate cancer in men undergoing repeat biopsy. *Urology,* 69(3), pp. 532-535.
[6] Payne, S.R., Serth, J., Schostak, M., Kamradt, J., Strauss, A., Thelen, P., Model, F., Day, J.K., Liebenberg, V., Morotti, A., Yamamura, S., Lograsso, J., Sledziewski, A. and Semjonow, A., 2009. DNA Methylation Biomarkers of Prostate Cancer: Confirmation of Candidates and Evidence Urine is the Most Sensitive Body Fluid for Non-Invasive Detection. *Prostate,* 69(12), pp. 1257-1269.
[7] Hamelin-Peyron, C., Vlaeminck-Guillem, V., Haidous, H., Schwall, G.P., Poznanovic, S., Gorius-Gallet, E., Michel, S., Larue, A., Guillotte, M., Ruffion, A., Choquet-Kastylevsky, G. and Ataman-Onal, Y., 2014. Prostate cancer biomarker annexin A3 detected in urines obtained following digital rectal examination presents antigenic variability. *Clinical Biochemistry,* 47(10-11), pp. 901-908.
[8] Yang, J., Bielenberg, D.R., Rodig, S.J., Doiron, R., Clifton, M.C., Kung, A.L., Strong, R.K., Zurakowski, D. and Moses, M.A., 2009. Lipocalin 2 promotes breast cancer progression. *Proceedings of the National Academy of Sciences of the United States of America,* 106(10), pp. 3913-3918.
[9] Gueraud, F., Peiro, G., Bernard, H., Alary, J., Creminon, C., Debrauwer, L., Rathahao, E., Drumare, M.F., Canlet, C., Wal, J.M. and Bories, G., 2006. Enzyme immunoassay for a urinary metabolite of 4-hydroxynonenal as a marker of lipid peroxidation. *Free Radical Biology and Medicine,* 40(1), pp. 54-62.
[10] Lin, H.J., Chen, S.T., Wu, H.Y., Hsu, H.C., Chen, M.F., Lee, Y.T., Wu, K.Y. and Chien, K.L., 2015. Urinary biomarkers of oxidative and nitrosative stress and the risk for incident stroke: A nested case-control study from a community-based cohort. *International Journal of Cardiology,* 183, pp. 214-220.
[11] Ming, X., Stein, T.P., Brimacombe, M., Johnson, W.G., Lambert, G.H. and Wagner, G.C., 2005. Increased excretion of a lipid peroxidation biomarker in autism. *Prostaglandins Leukotrienes and Essential Fatty Acids,* 73(5), pp. 379-384.
[12] Sardh, E., Harper, P., Andersson, D.E.H. and Floderus, Y., 2009. Plasma porphobilinogen as a sensitive biomarker to monitor the clinical and therapeutic course of acute intermittent porphyria attacks. *European Journal of Internal Medicine,* 20(2), pp. 201-207.
[13] Song, H.J., Chan, J. and Rovin, B.H., 2009. Induction of chemokine expression by adiponectin in vitro is isoform dependent. *Translational Research,* 154(1), pp. 18-26.
[14] Delmas, P.D., Schlemmer, A., Gineyts, E., Riis, B. and Christiansen, C., 1991. URINARY-EXCRETION OF PYRIDINOLINE CROSS-LINKS CORRELATES WITH BONE TURNOVER MEASURED ON ILIAC CREST BIOPSY IN PATIENTS WITH VERTEBRAL OSTEOPOROSIS. *Journal of Bone and Mineral Research,* 6(6), pp. 639-644.
[15] Devarajan, P., 2008. Proteomics for the investigation of acute kidney injury. *Proteomics in Nephrology - Towards Clinical Applications,* 160, pp. 1-16.
[16] Ito, H., Fujita, H. and Takahashi, T., 2008. Diagnostic biomarkers of diabetic nephropathy. *Expert opinion on medical diagnostics,* 2(2), pp. 161-169.
[17] McClintock, D.E., Ware, L.B., Eisner, M.D., Wickersham, N., Thompson, B.T., Matthay, M.A. and Natl Heart Lung Blood Inst, A.N., 2007. Higher urine nitric oxide is associated with improved outcomes in patients with acute lung injury. *American Journal of Respiratory and Critical Care Medicine,* 175(3), pp. 256-262.

[18] Adachi, J., Kumar, C., Zhang, Y., Olsen, J.V. and Mann, M., 2006. The human urinary proteome contains more than 1500 proteins, including a large proportion of membrane proteins. *Genome Biology,* 7(9).
[19] Ma, L.N., Wang, R., Han, Y., Sheng, S.L., Zhu, J.A., Ji, Z.J., Zhao, Z.W., Cao, Z.Q. and Wang, P.C., 2016. Development of a Novel Urine Alzheimer-Associated Neuronal Thread Protein ELISA Kit and Its Potential Use in the Diagnosis of Alzheimer's Disease. *Journal of Clinical Laboratory Analysis,* 30(4), pp. 308-314.
[20] Nkuipou-Kenfack, E., Zuerbig, P. and Mischak, H., 2017. The long path towards implementation of clinical proteomics: Exemplified based on CKD273. *Proteomics Clinical Applications,* 11(5-6).
[21] Rodriguez-Suarez, E., Siwy, J., Zurbig, P. and Mischak, H., 2014. Urine as a source for clinical proteome analysis: From discovery to clinical application. *Biochimica Et Biophysica Acta-Proteins and Proteomics,* 1844(5), pp. 884-898.
[22] Parker, C.E. and Borchers, C.H., 2014. Mass spectrometry based biomarker discovery, verification, and validation - Quality assurance and control of protein biomarker assays. *Molecular Oncology,* 8(4), pp. 840-858.
[23] Pan, S., Aebersold, R., Chen, R., Rush, J., Goodlett, D.R., McIntosh, M.W., Zhang, J. and Brentnall, T.A., 2009. Mass Spectrometry Based Targeted Protein Quantification: Methods and Applications. *Journal of Proteome Research,* 8(2), pp. 787-797.
[24] Kuzyk, M.A., Smith, D., Yang, J.C., Cross, T.J., Jackson, A.M., Hardie, D.B., Anderson, N.L. and Borchers, C.H., 2009. Multiple Reaction Monitoring-based, Multiplexed, Absolute Quantitation of 45 Proteins in Human Plasma. *Molecular & Cellular Proteomics,* 8(8), pp. 1860-1877.
[25] Yin, H.R., Zhang, L., Xie, L.Q., Huang, L.Y., Xu, Y., Cai, S.J., Yang, P.Y. and Lu, H.J., 2013. Hyperplex-MRM: A Hybrid Multiple Reaction Monitoring Method Using mTRAQ/iTRAQ Labeling for Multiplex Absolute Quantification of Human Colorectal Cancer Biomarker. *Journal of Proteome Research,* 12(9), pp. 3912-3919.
[26] Voisin, S.N., Krakovska, O., Matta, A., DeSouza, L.V., Romaschin, A.D., Colgan, T.J. and Siu, K.W.M., 2011. Identification of Novel Molecular Targets for Endometrial Cancer Using a Drill-Down LC-MS/MS Approach with iTRAQ. *Plos One,* 6(1).
[27] Fuzery, A.K., Levin, J., Chan, M.M. and Chan, D.W., 2013. Translation of proteomic biomarkers into FDA approved cancer diagnostics: issues and challenges. *Clinical proteomics,* 10(1), pp. 13-13.
[28] Renata, Y., Jassar, H., Katz, R., Hochberg, A., Nir, R.-R. and Klein-Kremer, A., 2013. Urinary concentration of cytokines in children with acute pyelonephritis. *European Journal of Pediatrics,* 172(6), pp. 769-774.
[29] Arneth, B.M., 2009. Clinical Significance of Measuring Prostate-Specific Antigen. *Labmedicine,* 40(8), pp. 487-491.
[30] Ng, T.K., Vasilareas, D., Mitterdorfer, A.J., Maher, P.O. and Lalak, A., 2005. Prostate cancer detection with digital rectal examination, prostate-specific antigen, transrectal ultrasonography and biopsy in clinical urological practice. *Bju International,* 95(4), pp. 545-548.
[31] Theodorescu, D., Schiffer, E., Bauer, H.W., Douwes, F., Eichhorn, F., Polley, R., Schmidt, T., Schoefer, W., Zuerbig, P., Good, D.M., Coon, J.J. and Mischak, H., 2008. Discovery and validation of urinary biomarkers for prostate cancer. *Proteomics Clinical Applications,* 2(4), pp. 556-570.
[32] Stuveling, E.M., Hillege, H.L., Bakker, S.J.L., Gans, R.O.B., de Jong, P.E. and de Zeeuw, D., 2003. C-reactive protein is associated with renal function abnormalities in a non-diabetic population. *Kidney International,* 63(2), pp. 654-661.
[33] Omasits, U., Ahrens, C.H., Muller, S. and Wollscheid, B., 2014. Protter: interactive protein feature visualization and integration with experimental proteomic data. Bioinformatics, pp. 884-886.
[34] Daughton, C.G., 2001. Emerging pollutants, and communicating the science of environmental chemistry and mass spectrometry: Pharmaceuticals in the environment. *Journal of the American Society for Mass Spectrometry,* 12(10), pp. 1067-1076.

[35] Phung, D., Mueller, J., Lai, F.Y., O'Brien, J., Dang, N., Morawska, L. and Thai, P.K., 2017. Can wastewater-based epidemiology be used to evaluate the health impact of temperature? - An exploratory study in an Australian population. *Environmental Research,* 156, pp. 113-119.
[36] Gao, J.F., O'Brien, J., Du, P., Li, X.Q., Ort, C., Mueller, J.F. and Thai, P.K., 2016. Measuring selected PPCPs in wastewater to estimate the population in different cities in China. *Science of the Total Environment,* 568, pp. 164-170.
[37] Burgard, D.A., Banta-Green, C. and Field, J.A., 2014. Working Upstream: How Far Can You Go with Sewage-Based Drug Epidemiology? *Environmental Science & Technology,* 48(3), pp. 1362-1368.
[38] Damien, D.A., Thomas, N., Helene, P., Sara, K. and Yves, L., 2014. First evaluation of illicit and licit drug consumption based on wastewater analysis in Fort de France urban area (Martinique, Caribbean), a transit area for drug smuggling. *Science of the Total Environment,* 490, pp. 970-978.
[39] Ort, C., van Nuijs, A.L.N., Berset, J.-D., Bijlsma, L., Castiglioni, S., Covaci, A., de Voogt, P., Emke, E., Fatta-Kassinos, D., Griffiths, P., Hernandez, F., Gonzalez-Marino, I., Grabic, R., Kasprzyk-Hordern, B., Mastroianni, N., Meierjohann, A., Nefau, T., Oestman, M., Pico, Y., Racamonde, I., Reid, M., Slobodnik, J., Terzic, S., Thomaidis, N. and Thomas, K.V., 2014. Spatial differences and temporal changes in illicit drug use in Europe quantified by wastewater analysis. *Addiction,* 109(8), pp. 1338-1352.
[40] Ryu, Y., Gracia-Lor, E., Bade, R., Baz-Lomba, J.A., Bramness, J.G., Castiglioni, S., Castrignano, E., Causanilles, A., Covaci, A., de Voogt, P., Hernandez, F., Kasprzyk-Hordern, B., Kinyua, J., McCall, A.K., Ort, C., Plosz, B.G., Ramin, P., Rousis, N.I., Reid, M.J. and Thomas, K.V., 2016. Increased levels of the oxidative stress biomarker 8-iso-prostaglandin F-2 alpha in wastewater associated with tobacco use. *Scientific Reports,* 6.
[41] Gracia-Lor, E., Castiglioni, S., Bade, R., Been, F., Castrignano, E., Covaci, A., Gonzalez-Marino, I., Hapeshi, E., Kasprzyk-Hordern, B., Kinyua, J., Lai, F.Y., Letzel, T., Lopardo, L., Meyer, M.R., O'Brien, J., Ramin, P., Rousis, N.I., Rydevik, A., Ryu, Y., Santos, M.M., Senta, I., Thomaidis, N.S., Veloutsou, S., Yang, Z.G., Zuccato, E. and Bijlsma, L., 2017. Measuring biomarkers in wastewater as a new source of epidemiological information: Current state and future perspectives. *Environment International,* 99, pp. 131-150.
[42] Castrignano, E., Lubben, A. and Kasprzyk-Hordern, B., 2016. Enantiomeric profiling of chiral drug biomarkers in wastewater with the usage of chiral liquid chromatography coupled with tandem mass spectrometry. *Journal of Chromatography A,* 1438, pp. 84-99.
[43] Depaolini, A.R., Fattore, E., Cappelli, F., Pellegrino, R., Castiglioni, S., Zuccato, E., Fanelli, R. and Davoli, E., 2016. Source discrimination of drug residues in wastewater: The case of salbutamol. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences,* 1023, pp. 62-67.
[44] Ryu, Y., Reid, M.J. and Thomas, K.V., 2015. Liquid chromatography-high resolution mass spectrometry with immunoaffinity clean-up for the determination of the oxidative stress biomarker 8-iso-prostaglandin F2alpha in wastewater. *Journal of Chromatography A,* 1409, pp. 146-151.
[45] Lai, F.Y., Bruno, R., Leung, H.W., Thai, P.K., Ort, C., Carter, S., Thompson, K., Lam, P.K.S. and Mueller, J.F., 2013. Estimating daily and diurnal variations of illicit drug use in Hong Kong: A pilot study of using wastewater analysis in an Asian metropolitan city. *Forensic Science International,* 233(1-3), pp. 126-132.
[46] Senta, I., Gracia-Lor, E., Borsotti, A., Zuccato, E. and Castiglioni, S., 2015. Wastewater analysis to monitor use of caffeine and nicotine and evaluation of their metabolites as biomarkers for population size assessment. *Water Research,* 74, pp. 23-33.
[47] Zheng, Q.D., Lin, J.G., Pei, W., Guo, M.X., Wang, Z. and Wang, D.G., 2017. Estimating nicotine consumption in eight cities using sewage epidemiology based on ammonia nitrogen equivalent population. *Science of the Total Environment,* 590, pp. 226-232.
[48] Baz-Lomba, J.A., Salvatore, S., Gracia-Lor, E., Bade, R., Castiglioni, S., Castrignano, E., Causanilles, A., Hernandez, F., Kasprzyk-Hordern, B., Kinyua, J., McCall, A.K., van Nuijs, A., Ort, C., Plosz, B.G., Ramin, P., Reid, M., Rousis, N.I., Ryu, Y., de Voogt, P., Bramness, J. and Thomas, K., 2016.

Comparison of pharmaceutical, illicit drug, alcohol, nicotine and caffeine levels in wastewater with sale, seizure and consumption data for 8 European cities. *Bmc Public Health,* 16.
[49] van Wel, J.H.P., Gracia-Lor, E., van Nuijs, A.L.N., Kinyua, J., Salvatore, S., Castiglioni, S., Bramness, J.G., Covaci, A. and Van Hal, G., 2016. Investigation of agreement between wastewater-based epidemiology and survey data on alcohol and nicotine use in a community. *Drug and Alcohol Dependence,* 162, pp. 170-175.
[50] van Nuijs, A.L.N., Covaci, A., Beyers, H., Bervoets, L., Blust, R., Verpooten, G., Neels, H. and Jorens, P.G., 2015. Do concentrations of pharmaceuticals in sewage reflect prescription figures? *Environmental Science and Pollution Research,* 22(12), pp. 9110-9118.
[51] Lai, F.Y., O'Brien, J., Bruno, R., Hall, W., Prichard, J., Kirkbride, P., Gartner, C., Thai, P., Carter, S., Lloyd, B., Burns, L. and Mueller, J., 2016. Spatial variations in the consumption of illicit stimulant drugs across Australia: A nationwide application of wastewater-based epidemiology. *Science of the Total Environment,* 568, pp. 810-818.
[52] Thomas, K.V., Bijlsma, L., Castiglioni, S., Covaci, A., Emke, E., Grabic, R., Hernandez, F., Karolak, S., Kasprzyk-Hordern, B., Lindberg, R.H., de Alda, M.L., Meierjohann, A., Ort, C., Pico, Y., Quintana, J.B., Reid, M., Rieckermann, J., Terzic, S., van Nuijs, A.L.N. and de Voogt, P., 2012. Comparing illicit drug use in 19 European cities through sewage analysis. *Science of the Total Environment,* 432, pp. 432-439.
[53] EMCDDA, 2016. *European Drug Report 2016: Trends and Developments.* Publications Office of the European Union, Luxembourg.
[54] EMCDDA, 2017. *European drug report 2017: trends and developments.* Belgium: Publications office of the European Union, Luxembourg.
[55] EMCDDA, 2015. *European drug report 2015: Trends and developments.* Spain: Publication office of the European Union, Luxembourg.
[56] EMCDDA, 2014. *European drug report 2014: trends and development.* Spain: Publications office of the European Union, Luxembourg.
[57] EMCDDA, 2013. *European monitoring report 2013: trends and developments.* Spain: Publications office of the European union, Luxenbourg.
[58] EMCDDA, 2011. *Annual report 2011: the state of the drug problem in Europe.* Luxembourg: Publications office of the European Union, Luxembourg.
[59] EMCDDA, 2012. *Annual report 2012: the state of the drug problem in Europe.* Luxembourg: Publications office of the European Union, Luxembourg.
[60] Gracia-Lor, E., Rousis, N.I., Zuccato, E., Bade, R., Baz-Lomba, J.A., Castrignano, E., Causanilles, A., Hernandez, F., Kasprzyk-Hordern, B., Kinyua, J., McCall, A.K., van Nuijs, A.L.N., Plosz, B.G., Ramin, P., Ryu, Y., Santos, M.M., Thomas, K., de Voogt, P., Yang, Z.G. and Castiglioni, S., 2017. Estimation of caffeine intake from analysis of caffeine metabolites in wastewater. *Science of the Total Environment,* 609, pp. 1582-1588.
[61] Ryu, Y., Barcelo, D., Barron, L.P., Bijlsma, L., Castiglioni, S., de Voogt, P., Emke, E., Hernandez, F., Lai, F.Y., Lopes, A., de Alda, M.L., Mastroianni, N., Munro, K., O'Brien, J., Ort, C., Plosz, B.G., Reid, M.J., Yargeau, V. and Thomas, K.V., 2016. Comparative measurement and quantitative risk assessment of alcohol consumption through wastewater-based epidemiology: An international study in 20 cities. *Science of the Total Environment,* 565, pp. 977-983.
[62] Castiglioni, S., Senta, I., Borsotti, A., Davoli, E. and Zuccato, E., 2015. A novel approach for monitoring tobacco use in local communities by wastewater analysis. *Tobacco Control,* 24(1), pp. 38-42.
[63] Wu, C., Chen, S.T., Peng, K.H., Cheng, T.J. and Wu, K.Y., 2016. Concurrent quantification of multiple biomarkers indicative of oxidative stress status using liquid chromatography-tandem mass spectrometry. *Analytical Biochemistry,* 512, pp. 26-35.
[64] Rousis, N.I., Gracia-Lor, E., Zuccato, E., Bade, R., Baz-Lomba, J.A., Castrignano, E., Causanilles, A., Covaci, A., de Voogt, P., Hernandez, F., Kasprzyk-Hordern, B., Kinyua, J., McCall, A.K., Plosz, B.G.,

Ramin, P., Ryu, Y., Thomas, K.V., van Nuijs, A., Yang, Z.G. and Castiglioni, S., 2017. Wastewater-based epidemiology to assess pan-European pesticide exposure. *Water Research,* 121, pp. 270-279.
[65] Lopardo, L., Petrie, B., Proctor, K., Youdan, J., Barden, R. and Kasprzyk-Hordern, B., 2019. Estimation of community-wide exposure to bisphenol A via water fingerprinting. *Environ Int,* 125, pp. 1-8.
[66] Lopardo, L., Adams, D., Cummins, A. and Kasprzyk-Hordern, B., 2018. Verifying community-wide exposure to endocrine disruptors in personal care products - In quest for metabolic biomarkers of exposure via in vitro studies and wastewater-based epidemiology. *Water Research,* 143, pp. 117-126.
[67] Fattore, E., Davoli, E., Castiglioni, S., Bosetti, C., Depaolini, A.R., Marzona, I., Zuccato, E. and Fanelli, R., 2016. y Wastewater-based epidemiological evaluation of the effect of air pollution on short-acting beta-agonist consumption for acute asthma treatment. *Environmental Research,* 150, pp. 106-111.
[68] Bijlsma, L., Botero-Coy, A.M., Rincon, R.J., Penuela, G.A. and Hernandez, F., 2016. Estimation of illicit drug use in the main cities of Colombia by means of urban wastewater analysis. *Science of the Total Environment,* 565, pp. 984-993.
[69] Causanilles, A., Ruepert, C., Ibanez, M., Emke, E., Hernandez, F. and de Voogt, P., 2017. Occurrence and fate of illicit drugs and pharmaceuticals in wastewater from two wastewater treatment plants in Costa Rica. *Science of the Total Environment,* 599, pp. 98-107.
[70] Du, P., Zhou, Z.L., Bai, Y., Xu, Z.Q., Gao, T.T., Fu, X.F. and Li, X.Q., 2017. Estimating heroin abuse in major Chinese cities through wastewater-based epidemiology. *Science of the Total Environment,* 605, pp. 158-165.
[71] Du, P., Li, K.Y., Li, J., Xu, Z.Q., Fu, X.F., Yang, J., Zhang, H.F. and Li, X.Q., 2015. Methamphetamine and ketamine use in major Chinese cities, a nationwide reconnaissance through sewage-based epidemiology. *Water Research,* 84, pp. 76-84.
[72] Kim, K.Y., Lai, F.Y., Kim, H.Y., Thai, P.K., Mueller, J.F. and Oh, J.E., 2015. The first application of wastewater-based drug epidemiology in five South Korean cities. *Science of the Total Environment,* 524, pp. 440-446.
[73] Mastroianni, N., Lopez-Garcia, E., Postigo, C., Barcelo, D. and de Alda, M.L., 2017. Five-yearmonitoring of 19 illicit and legal substances of abuse at the inlet of a wastewater treatment plant in Barcelona (NE Spain) and estimation of drug consumption patterns and trends. *Science of the Total Environment,* 609, pp. 916-926.
[74] Tscharke, B.J., Chen, C., Gerber, J.P. and White, J.M., 2016. Temporal trends in drug use in Adelaide, South Australia by wastewater analysis. *Science of the Total Environment,* 565, pp. 384-391.
[75] Baz-Lomba, J.A., Reid, M.J. and Thomas, K.V., 2016. Target and suspect screening of psychoactive substances in sewage-based samples by UHPLC-QTOF. *Analytica Chimica Acta,* 914, pp. 81-90.
[76] Klupczynska, A., Derezinski, P., Krysztofiak, J. and Kokot, Z.J., 2016. Estimation of drug abuse in 9 Polish cities by wastewater analysis. *Forensic Science International,* 260, pp. 14-21.
[77] Krizman, I., Senta, I., Ahel, M. and Terzic, S., 2016. Wastewater-based assessment of regional and temporal consumption patterns of illicit drugs and therapeutic opioids in Croatia. *Science of the Total Environment,* 566, pp. 454-462.
[78] Subedi, B. and Kannan, K., 2014. Mass Loading and Removal of Select Illicit Drugs in Two Wastewater Treatment Plants in New York State and Estimation of Illicit Drug Usage in Communities through Wastewater Analysis. *Environmental Science & Technology,* 48(12), pp. 6661-6670.
[79] Vuori, E., Happonen, M., Gergov, M., Nenonen, T., Jarvinen, A., Ketola, R.A. and Vahala, R., 2014. Wastewater analysis reveals regional variability in exposure to abused drugs and opioids in Finland. *Science of the Total Environment,* 487, pp. 688-695.
[80] Gatidou, G., Kinyua, J., van Nuijs, A.L.N., Gracia-Lor, E., Castiglioni, S., Covaci, A. and Stasinakis, A.S., 2016. Drugs of abuse and alcohol consumption among different groups of population on the

Greek Island of Lesvos through sewage-based epidemiology. *Science of the Total Environment,* 563, pp. 633-640.
[81] Boogaerts, T., Covaci, A., Kinyua, J., Neels, H. and van Nuijs, A.L.N., 2016. Spatial and temporal trends in alcohol consumption in Belgian cities: A wastewater-based approach. *Drug and Alcohol Dependence,* 160, pp. 170-176.
[82] Lai, F.Y., Been, F., Covaci, A. and van Nuijs, A.L.N., 2017. Novel Wastewater-Based Epidemiology Approach Based on Liquid Chromatography Tandem Mass Spectrometry for Assessing Population Exposure to Tobacco-Specific Toxicants and Carcinogens. *Analytical Chemistry,* 89(17), pp. 9268-9278.
[83] Rodriguez-Alvarez, T., Rodil, R., Rico, M., Cela, R. and Quintana, J.B., 2014. Assessment of Local Tobacco Consumption by Liquid Chromatography-Tandem Mass Spectrometry Sewage Analysis of Nicotine and Its Metabolites, Cotinine and trans-3 '-Hydroxycotinine, after Enzymatic Deconjugation. *Analytical Chemistry,* 86(20), pp. 10274-10281.
[84] Tscharke, B., White, J.M. and Gerber, J.P., 2016. Estimates of tobacco use by wastewater analysis of anabasine and anatabine. *Drug Testing and Analysis,* 8(7), pp. 702-707.
[85] Atwood, B.K., Huffman, J., Straiker, A. and Mackie, K., 2010. JWH018, a common constituent of 'Spice' herbal blends, is a potent and efficacious cannabinoid CB1 receptor agonist. *British Journal of Pharmacology,* 160(3), pp. 585-593.
[86] Gao, T.T., Du, P., Xu, Z.Q. and Li, X.Q., 2017. Occurrence of new psychoactive substances in wastewater of major Chinese cities. *Science of the Total Environment,* 575, pp. 963-969.
[87] Senta, I., Krizman, I., Ahel, M. and Terzic, S., 2015. Multiresidual analysis of emerging amphetamine-like psychoactive substances in wastewater and river water. *Journal of Chromatography A,* 1425, pp. 204-212.
[88] Kinyua, J., Covaci, A., Maho, W., McCall, A.K., Neels, H. and Nuijs, A.L.N., 2015. Sewage-based epidemiology in monitoring the use of new psychoactive substances: Validation and application of an analytical method using LC-MS/MS. *Drug Testing and Analysis,* 7(9), pp. 812-818.
[89] Wang, C.C., Hou, L.L., Li, J., Xu, Z.Q., Gao, T.T., Yang, J., Zhang, H.F., Li, X.Q. and Du, P., 2017. Occurrence of diazepam and its metabolites in wastewater and surface waters in Beijing. *Environmental Science and Pollution Research,* 24(18), pp. 15379-15389.
[90] Yuan, S.F., Liu, Z.H., Huang, R.P., Yin, H. and Dang, Z., 2016. Levels of six antibiotics used in China estimated by means of wastewater-based epidemiology. *Water Science and Technology,* 73(4), pp. 769-775.
[91] Sims, N., Rice, J. and Kasprzyk-Hordern, B., 2019. An ultra-high-performance liquid chromatography tandem mass spectrometry method for oxidative stress biomarker analysis in wastewater. *Analytical and Bioanalytical Chemistry,* 411(11), pp. 2261-2271.
[92] Choi, P.M., O'Brien, J.W., Li, J.Y., Jiang, G.M., Thomas, K.V. and Mueller, J.F., 2018. Population histamine burden assessed using wastewater-based epidemiology: The association of 1,4-methylimidazole acetic acid and fexofenadine. *Environment International,* 120, pp. 172-180.
[93] Lopardo, L., Cummins, A., Rydevik, A. and Kasprzyk-Hordern, B., 2017. New Analytical Framework for Verification of Biomarkers of Exposure to Chemicals Combining Human Biomonitoring and Water Fingerprinting. *Analytical Chemistry,* 89(13), pp. 7232-7239.
[94] Gregorich, Z.R. and Ge, Y., 2014. Top-down proteomics in health and disease: Challenges and opportunities. *Proteomics,* 14(10), pp. 1195-1210.
[95] Fortin, T., Salvador, A., Charrier, J.P., Lenz, C., Bettsworth, F., Lacoux, X., Choquet-Kastylevsky, G. and Lemoine, J., 2009. Multiple Reaction Monitoring Cubed for Protein Quantification at the Low Nanogram/Milliliter Level in Nondepleted Human Serum. *Analytical Chemistry,* 81(22), pp. 9343-9352.
[96] Kuhn, E., Wu, J., Karl, J., Liao, H., Zolg, W. and Guild, B., 2004. Quantification of C-reactive protein in the serum of patients with rheumatoid arthritis using multiple reaction monitoring mass spectrometry and C-13-labeled peptide standards. *Proteomics,* 4(4), pp. 1175-1186.

[97] Shi, T.J., Gao, Y.Q., Quek, S.I., Fillmore, T.L., Nicora, C.D., Su, D., Zhao, R., Kagan, J., Srivastava, S., Rodland, K.D., Liu, T., Smith, R.D., Chan, D.W., Camp, D.G., Liu, A.Y. and Qian, W.J., 2014. A Highly Sensitive Targeted Mass Spectrometric Assay for Quantification of AGR2 Protein in Human Urine and Serum. *Journal of Proteome Research,* 13(2), pp. 875-882.
[98] Shi, T.J., Fillmore, T.L., Gao, Y.Q., Zhao, R., He, J.T., Schepmoes, A.A., Nicora, C.D., Wu, C.C., Chambers, J.L., Moore, R.J., Kagan, J., Srivastava, S., Liu, A.Y., Rodland, K.D., Liu, T., Camp, D.G., Smith, R.D. and Qian, W.J., 2013. Long-Gradient Separations Coupled with Selected Reaction Monitoring for Highly Sensitive, Large Scale Targeted Protein Quantification in a Single Analysis. *Analytical Chemistry,* 85(19), pp. 9196-9203.
[99] Liu, T., Hossain, M., Schepmoes, A.A., Fillmore, T.L., Sokoll, L.J., Kronewitter, S.R., Izmirlian, G., Shi, T., Qian, W.-J., Leach, R.J., Thompson, I.M., Chan, D.W., Smith, R.D., Kagan, J., Srivastava, S., Rodland, K.D. and Camp, D.G., II, 2012. Analysis of serum total and free PSA using immunoaffinity depletion coupled to SRM: correlation with clinical immunoassay tests. *Journal of Proteomics,* 75(15), pp. 4747-4757.
[100] Rodriguez-Suarez, E. and Whetton, A.D., 2013. The application of quantification techniques in proteomics for biomedical research. *Mass Spectrometry Reviews,* 32(1), pp. 1-26.
[101] Janecki, D.J., Bemis, K.G., Tegeler, T.J., Sanghani, P.C., Zhai, L., Hurley, T.D., Bosron, W.F. and Wang, M., 2007. A multiple reaction monitoring method for absolute quantification of the human liver alcohol dehydrogenase ADH1C1 isoenzyme. *Analytical Biochemistry,* 369(1), pp. 18-26.
[102] Simpson, D.M. and Beynon, R.J., 2012. QconCATs: design and expression of concatenated protein standards for multiplexed protein quantification. *Analytical and Bioanalytical Chemistry,* 404(4), pp. 977-989.
[103] Bizzarri, M., Cavaliere, C., Foglia, P., Guarino, C., Samperi, R. and Lagana, A., 2008. A label-free method based on MALDI-TOF mass spectrometry for the absolute quantitation of troponin T in mouse cardiac tissue. *Analytical and Bioanalytical Chemistry,* 391(5), pp. 1969-1976.
[104] Resta, D., Brambilla, F. and Arnoldi, A., 2012. HPLC-Chip-Multiple Reaction Monitoring (MRM) method for the label-free absolute quantification of gamma-conglutin in lupin: Proteotypic peptides and standard addition method. *Food Chemistry,* 131(1), pp. 126-133.
[105] Rampoldi, L., Scolari, F., Amoroso, A., Ghiggeri, G. and Devuyst, O., 2011. The rediscovery of uromodulin (Tamm-Horsfall protein): from tubulointerstitial nephropathy to chronic kidney disease. *Kidney International,* 80(4), pp. 338-347.
[106] Slagter, M.H., Gooren, L.J.G., de Ronde, W., Soosaipillai, A., Scorilas, A., Giltay, E.J., Paliouras, M. and Diamandis, E.P., 2006. Serum and urine tissue kallikrein concentrations in male-to-female transsexuals treated with antiandrogens and estrogens. *Clinical Chemistry,* 52(7), pp. 1356-1365.
[107] Mannello, F., Condemi, L., Cardinali, A., Bianchi, G. and Gazzanelli, G., 1998. High concentrations of prostate-specific antigen in urine of women receiving oral contraceptives. *Clinical Chemistry,* 44(1), pp. 181-183.
[108] Wittke, S., Schiffer, E. and Bauer, H.W., 2007. Capillary electrophoresis coupled to mass spectrometry for proteome analysis. An innovative diagnostic method for prostate and bladder cancer. *Urologe,* 46(7), pp. 733-739.
[109] Roilides, E., Papachristou, F., Gioulekas, E., Tsaparidou, S., Karatzas, N., Sotiriou, J. and Tsiouris, J., 1999. Increased urine interleukin-6 concentrations correlate with pyelonephritic changes on Tc-99m-dimercaptosuccinic acid scans in neonates with urinary tract infections. *Journal of Infectious Diseases,* 180(3), pp. 904-907.
[110] Taha, A.S., Grant, V. and Kelly, R.W., 2003. Urinalysis for interleukin-8 in the non-invasive diagnosis of acute and chronic inflammatory diseases. *Postgraduate Medical Journal,* 79(929), pp. 159-163.
[111] Simon, R., Lemoine, J., Fonbonne, C., Jaffuel, A., Leonard, J.-F., Gautier, J.-C., Pasquier, O. and Salvador, A., 2014. Absolute quantification of podocin, a potential biomarker of glomerular injury in human urine, by liquid chromatography-multiple reaction monitoring cubed mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis,* 94, pp. 84-91.

[112] Garovic, V.D., Craici, I.M., Wagner, S.J., White, W.M., Brost, B.C., Rose, C.H., Grande, J.P. and Barnidge, D.R., 2013. Mass spectrometry as a novel method for detection of podocyturia in pre-eclampsia. *Nephrology Dialysis Transplantation,* 28(6), pp. 1555-1561.
[113] Hammond, T.G., Moes, S., Youhanna, S., Jennings, P., Devuyst, O., Odermatt, A. and Jeno, P., 2016. Development and characterization of a pseudo multiple reaction monitoring method for the quantification of human uromodulin in urine. *Bioanalysis,* 8(12), pp. 1279-1296.
[114] Selevsek, N., Matondo, M., Sanchez Carbayo, M., Aebersold, R. and Domon, B., 2011. Systematic quantification of peptides/proteins in urine using selected reaction monitoring. *Proteomics,* 11(6), pp. 1135-1147.
[115] Ray, S., Patel, S.K., Kumar, V., Damahe, J. and Srivastava, S., 2014. Differential expression of serum/plasma proteins in various infectious diseases: Specific or nonspecific signatures. *Proteomics Clinical Applications,* 8(1-2), pp. 53-72.
[116] Black, S., Kushner, I. and Samols, D., 2004. C-reactive Protein. *The Journal of biological chemistry,* 279(47), p. 48487.
[117] Jacobson, S.H., Hylander, B., Wretlind, B. and Brauner, A., 1994. INTERLEUKIN-6 AND INTERLEUKIN-8 IN SERUM AND URINE IN PATIENTS WITH ACUTE PYELONEPHRITIS IN RELATION TO BACTERIAL-VIRULENCE-ASSOCIATED TRAITS AND RENAL-FUNCTION. *Nephron,* 67(2), pp. 172-179.
[118] Hedges, S., Stenqvist, K., Lidinjanson, G., Martinell, J., Sandberg, T. and Svanborg, C., 1992. COMPARISON OF URINE AND SERUM CONCENTRATIONS OF INTERLEUKIN-6 IN WOMEN WITH ACUTE PYELONEPHRITIS OR ASYMPTOMATIC BACTERIURIA. *Journal of Infectious Diseases,* 166(3), pp. 653-656.
[119] Reyes-Thomas, J., Blanco, I. and Putterman, C., 2011. Urinary Biomarkers in Lupus Nephritis. *Clinical Reviews in Allergy & Immunology,* 40(3), pp. 138-150.
[120] Tashiro, K., Koyanagi, I., Saitoh, A., Shimizu, A., Shike, T., Ishiguro, C., Koizumi, M., Funabiki, K., Horikoshi, S., Shirato, I. and Tomino, Y., 2002. Urinary levels of monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8), and renal injuries in patients with type 2 diabetic nephropathy. *Journal of Clinical Laboratory Analysis,* 16(1), pp. 1-4.
[121] Petrie, B., Youdan, J., Barden, R. and Kasprzyk-Hordern, B., 2016. Multi-residue analysis of 90 emerging contaminants in liquid and solid environmental matrices by ultra-high-performance liquid chromatography tandem mass spectrometry. *Journal of Chromatography A,* 1431, pp. 64-78.
[122] Simon, R., Passeron, S., Lemoine, J. and Salvador, A., 2014. Hydrophilic interaction liquid chromatography as second dimension in multidimensional chromatography with an anionic trapping strategy: Application to prostate-specific antigen quantification. *Journal of Chromatography A,* 1354, pp. 75-84.
[123] Prakash, A., Rezai, T., Krastins, B., Sarracino, D., Athanas, M., Russo, P., Zhang, H., Tian, Y., Li, Y., Kulasingam, V., Drabovich, A., Smith, C.R., Batruch, I., Oran, P.E., Fredolini, C., Luchini, A., Liotta, L., Petricoin, E., Diamandis, E.P., Chan, D.W., Nelson, R. and Lopez, M.F., 2012. Interlaboratory Reproducibility of Selective Reaction Monitoring Assays Using Multiple Upfront Analyte Enrichment Strategies. *Journal of Proteome Research,* 11(8), pp. 3986-3995.
[124] Buszewski, B. and Noga, S., 2012. Hydrophilic interaction liquid chromatography (HILIC)-a powerful separation technique. *Analytical and Bioanalytical Chemistry,* 402(1), pp. 231-247.
[125] Khan, U. and Nicell, J.A., 2011. Refined sewer epidemiology mass balances and their application to heroin, cocaine and ecstasy. *Environment International,* 37(7), pp. 1236-1252.
[126] Gracia-Lor, E., Zuccato, E. and Castiglioni, S., 2016. Refining correction factors. for back-calculation of illicit drug use. *Science of the Total Environment,* 573, pp. 1648-1659.
[127] Thai, P.K., O'Brien, J.W., Tscharke, B.J. and Mueller, J.F., 2019. Analyzing Wastewater Samples Collected during Census To Determine the Correction Factors of Drugs for Wastewater-Based Epidemiology: The Case of Codeine and Methadone. *Environmental Science & Technology Letters,* 6(5), pp. 265-269.
[128] Goulding, N., Hickman, M., Reid, M., Amundsen, E.J., Baz-Lomba, J.A., O'Brien, J.W., Tscharke, B.J., deVoogt, P., Emke, E., Kuijpers, W., Hall, W. and Jones, H.E., 2020. A comparison of trends in

wastewater-based data and traditional epidemiological indicators of stimulant consumption in three locations. *Addiction,* 115(3), pp. 462-472.
[129] Jones, H.E., Hickman, M., Kasprzyk-Hordern, B., Welton, N.J., Baker, D.R. and Ades, A.E., 2014. Illicit and pharmaceutical drug consumption estimated via wastewater analysis. Part B: Placing back-calculations in a formal statistical framework. *Science of the Total Environment,* 487, pp. 642-650.
[130] Horsewood, P., 1988. Specific Proteins Analysis by Nephelometry and Turbidimetry. In: N. T.T., ed. *Nonisotopic Immunoassay.* Springer, Boston, MA.
[131] Lin, A.V., 2015. Direct ELISA. In: R. Hnasko, ed. *ELISA. Methods in Molecular Biology.* Humana press, New York, NY, pp. 61-67.
[132] Lin, A.V., 2015. Indirect ELISA. In: R. Hnasko, ed. *ELISA. Methods in Molecular Biology.* Humana press, New York, NY, pp. 51-59.
[133] Chen, E.I., Cociorva, D., Norris, J.L. and Yates, J.R., III, 2007. Optimization of mass spectrometry-compatible surfactants for shotgun proteomics. *Journal of Proteome Research,* 6(7), pp. 2529-2538.
[134] Aebersold, R. and Mann, M., 2003. Mass spectrometry-based proteomics. *Nature,* 422(6928), pp. 198-207.
[135] Davalieva, K., Kiprijanovska, S., Komina, S., Petrusevska, G., Zografska, N.C. and Polenakovic, M., 2015. Proteomics analysis of urine reveals acute phase response proteins as candidate diagnostic biomarkers for prostate cancer. *Proteome Science,* 13.
[136] Becker, C.H. and Bern, M., 2011. Recent developments in quantitative proteomics. *Mutation Research-Genetic Toxicology and Environmental Mutagenesis,* 722(2), pp. 171-182.
[137] Boersema, P.J., Mohammed, S. and Heck, A.J.R., 2008. Hydrophilic interaction liquid chromatography (HILIC) in proteomics. *Analytical and Bioanalytical Chemistry,* 391(1), pp. 151-159.
[138] Choi, Y.S., 2012. Reaching for the deep proteome: Recent nano liquid chromatography coupled with tandem mass spectrometry-based studies on the deep proteome. *Archives of Pharmacal Research,* 35(11), pp. 1861-1870.
[139] Zauner, G., Koeleman, C.A.M., Deelder, A.M. and Wuhrer, M., 2010. Protein glycosylation analysis by HILIC-LC-MS of Proteinase K-generated N- and O-glycopeptides. *Journal of Separation Science,* 33(6-7), pp. 903-910.
[140] Fortin, T., Salvador, A., Charrier, J.P., Lenz, C., Lacoux, X., Morla, A., Choquet-Kastylevsky, G. and Lemoine, J., 2009. Clinical Quantitation of Prostate-specific Antigen Biomarker in the Low Nanogram/Milliliter Range by Conventional Bore Liquid Chromatography-Tandem Mass Spectrometry (Multiple Reaction Monitoring) Coupling and Correlation with ELISA Tests. *Molecular & Cellular Proteomics,* 8(5), pp. 1006-1015.
[141] Vegvari, A., Sjodin, K., Rezeli, M., Malm, J., Lilja, H., Laurell, T. and Marko-Varga, G., 2013. Identification of a Novel Proteoform of Prostate Specific Antigen (SNP-L132I) in Clinical Samples by Multiple Reaction Monitoring. *Molecular & Cellular Proteomics,* 12(10), pp. 2761-2773.
[142] Lesur, A., Varesio, E., Domon, B. and Hopfgartner, G., 2012. Peptides Quantification by Liquid Chromatography with Matrix-Assisted Laser Desorption/Ionization and Selected Reaction Monitoring Detection. *Journal of Proteome Research,* 11(10), pp. 4972-4982.

Addendum

When writing this paper for publication the decision was made to focus solely on WBE for collecting data on public health. Whilst WBE is a very powerful technique it cannot and should not stand alone. As was discussed in chapter one, non-wastewater sources of data, such as local prescription data, are important for giving context to the results of WBE investigations and for the important epidemiological work that takes place after wastewater analysis. For example, without local prescription data the calculation of heroin in chapter one would have to rely on using either only morphine or only O-6-MAM loads in wastewater, both of which are problematic [70; 125]. This epidemiological work can include determining correction factors for better analysis [126; 127] but also modelling drug usage with the community [128; 129]. This type of work is essential for the development of WBE going forwards, particularly for biomarkers of human health, like proteins and biomarkers of oxidative stress, in determining when public health intervention is necessary.

Likewise there was not the opportunity to discuss protein analysis in depth, particularly how it differs from proteomics and the specifics of protein analysis by LC-MS/MS. In brief, proteomics is the study of all proteins in a system, like a cell, humans or wastewater, whilst protein analysis refers simply to the analysis of one or more proteins. Within literature there are a variety of different methods used for the analysis of proteins in clinical samples and is not as cut and dry as outlined in the chapter, which briefly outlined a move from analysis using ELISA to analysis using LC-MS/MS. Firstly, there are physical methods of analysing proteins via techniques like nephelometry, which measures the amount of light scattered by an antibody-antigen complex to determine the concentration of either the antigen or antibody by comparison to a calibration curve of known concentrations [130]. Nephelometry was used to quantify urinary serum albumin and urinary CRP in the city-wide public health study discussed in the chapter [32]. The popularity of the technique comes from its reliability and the ability for automation, making it an essential workhorse for clinical analysis, however it is not as sensitive as other antibody based techniques such as ELISA, which is regarded as the gold standard for clinical analysis [24]. ELISA differs from nephelometry by using an antibody or antigen that is attached to a surface and adding a reagent that measures the formation of the antibody-antigen complex [131; 132]. The analysis of interleukins IL-6 and IL-8 in literature has almost exclusively been performed by ELISA [28; 109; 110; 120]. The advantages of ELISA include its ability to generate sensitive, quantitative results in only a few hours, which is very important for ensuring rapid disease diagnosis [131; 132], particularly where it can be used to assess disease outcomes as well [109]. Additionally, the use of multi-well plates makes it possible to analyse several clinical samples at once, which increasing sample throughput compared to nephelometry.

Other non-MS, non-antibody based methods of analysis do also exist, such as gel electrophoresis combined with staining [133] but these methods have largely been supplanted by more sensitive

methods of protein/peptide detection, although examples of gel electrophoresis coupled with mass spectrometry do appear in literature [134; 135]. The advantages of using liquid chromatography over electrophoresis, aside from its ability to be directly coupled to MS, come from the ability to mix different stationary and mobile phases together in order to create a wide variety of separation conditions to aid analyte detection. In contrast gel electrophoresis can struggle to detect low abundance proteins due to a limited dynamic range [134] that leads to overlapping peaks, essentially biasing the technique towards the most abundant proteins in the sample. The most common form of LC method described in literature is one that uses reverse-phase (RP) chromatography with a water/methanol or water/acetonitrile mobile phase and a C_{18} stationary phase [136]. However, alternatives like hydrophilic interaction liquid chromatrography (HILIC) [124; 137] or ion chromatography [124] can be used to selective separate different peptides. Multiple or different stationary phases can also be linked together to provide two-dimensional separation. For example, by using two reverse-phase columns and two different pH gradients it was possible to selectively separate phosphopeptides from other peptides, in a similar manner to the electrophoresis technique of isoelectric focussing that separates analytes by pH [138]. Likewise, RP and HILIC are often combined together to give greater analytical sensitivity by selectively trapping analytes, separating them from matrix, in the first dimension and then separating them from each other in the second [122; 137; 139]. HILIC is particularly suited for use as the second dimension when coupled to MS [122], as the mobile phase by necessity contains a large percentage of organic solvents, which can make it easier to ionise analytes in the MS source.

Despite the supposed ascendency of MS over ELISA for protein and peptide analysis, a lot of method LC-MS/MS method development is focused on trying to develop methods as sensitive or as rapid as ELISA [24; 98]. Despite this, the advantages of LC-MS/MS lie in its ability to more easily allow for multi-biomarker assays through a combination of easier sample multiplexing and lower method development costs [22; 23; 99; 140], due to a lack of need for antibody development, which has been estimated to cost >$100,000 per antibody [22]. LC-MS/MS was used to quantify the following biomarkers proposed earlier in the chapter: podocin [112], uromodulin [113] and PSA [95; 99; 141]. However, whilst LC-MS/MS may be used to move away from antibody based methods of detection this does not mean that antibodies are not still used, with several papers making use of antibody based affinity columns to either deplete (i.e. remove matrix) or enrich (i.e. concentrate an analyte) samples prior to detection [99; 140].

For public health analysis the main advantage of using LC-MS/MS for protein analysis is that the same range of instrumentation can be used to analyse smaller molecules and peptides, with many WBE biomarkers, CEC and peptides analysed using the same range of RP columns coupled with triple quadrupole or hybrid time of flight instrumentation and ESI. However, others types of detector and ionisation are also common within protein analysis including ion traps, Orbitrap type instruments

and the use MALDI. MALDI can often be seen coupled to gel based and offline LC methods of separation [23; 134-136; 142] as well as being used for the analysis of intact proteins [94]. Likewise, whilst affinity columns may be commonly used in clinical analysis they are likely to be less important for public health analysis. Firstly, the low protein concentration of wastewater makes immunodepletion unnecessary and secondly, the use of affinity columns to enrich samples removes the main advantage of LC-MS/MS over antibody based methods of detection; namely its ability to analyse multiple biomarkers at once.

Chapter five introduction

The previous chapter laid out the foundation of why urinary proteins should be considered for analysis in wastewater, particularly for their ability to measure public health using currently utilised methods of analysis. This formed the initial discussion of the third thesis aim of exploring proteins as potential new biomarkers of public health.

This chapter continues to explore this aim by practically examining the steps required to analyse proteins in wastewater, including the selection of potential biomarkers and the development of a digestion and analytical methodology for use in wastewater.

Initially, the chapter outlines enzyme digest conditions used in literature in order to identify what makes an effective and robust digestion method, which is crucial for reproducibly producing predicted peptides in wastewater. This is then followed by an assessment of which biomarkers from the previous chapter are most likely to be detected in wastewater, and should therefore be investigated first. This included the identification of human specific peptides from in-silico digestion and the estimation of wastewater protein concentrations. The bulk of the paper focuses on the step by step development of an enzyme digestion methodology for use in buffer and then wastewater and its subsequent use to identify human proteins via LC-MS/MS.

Development of an enzymatic digestion method for use in wastewater for the analysis of proteins of disease by hydrophilic interaction liquid chromatography coupled to triple quadrupole mass spectrometry

Abstract

Proteins were identified in the previous chapter as potential biomarkers of public health as by measuring urinary proteins that are biomarkers for human disease it would be possible to directly measure public health. Additionally, by using bottom-up proteomics to measure peptides it would be possible to use the same techniques currently used to analyse other public health biomarkers in wastewater-based epidemiology (WBE), which would make analysis of urinary proteins more readily accessible to the wider analytical community. However, wastewater is a challenging analytical matrix, combining the low protein concentration of urine, which is further reduced by dilution in the sewer system, the high matrix complexity of blood, and the contribution of non-human proteins to the wastewater proteome. This chapter details the development of a methodology that allowed for the detection of a human specific protein biomarker in wastewater using hydrophilic interaction liquid chromatography (HILIC) coupled to a triple quadrupole (QQQ) mass spectrometer. Additionally, the chapter explored the considerations that must be made when selecting biomarkers to ensure human specificity. An analysis of literature was used to identify the most common reagents used during enzyme digestion and was used to develop a simplified digestion method that did not use expensive reagents or difficult to remove surfactants. In-silico digests of five potential protein biomarkers were used to identify human specific peptides, as well as pseudo-specific peptides shared with non-native primates. Data from previous chapters was used to estimate the wastewater concentration of each of the five biomarkers and showed that the most abundant peptides should be detectable using current WBE techniques. The final method digested used 100 mL of wastewater and led to the potential detection of one peptide of C-Reactive Protein (CRP) in wastewater, which would require the use of a synthetic analogue to confirm. This represented the first tentative steps required for the development of a robust WBE-compatible protein analysis method. The in-silico digestion and wastewater concentration estimation carried out here should provide an excellent resource for other researchers interested in the wastewater proteome, allowing for the identification of species-specific biomarkers that are accessible to current analytical techniques.

1. Introduction

1.1 Public health monitoring

The use of analytical science for monitoring individual health is well established, with its utility and general purpose outlined in chapter four [1]. In brief, molecular diagnostics of human health has long been used for the analysis of individual health but techniques like wastewater-based epidemiology (WBE) seek to apply these methods to analysing public health. Currently, most WBE biomarkers are biomarkers of drugs of abuse or pharmaceuticals, although it should be noted that some endogenous human biomarkers have been considered [2-5]. It can be argued that this largely consumption-based approach gives only a limited understanding of public health, as it focuses on only the population that is consuming a biomarker and not the wider population. Whilst it is understandable that a less healthy population will consume more pharmaceuticals, drugs of abuse or other WBE biomarkers, this is still a passive approach to monitoring human health as it cannot say how what was consumed yesterday will affect public health tomorrow. However, by analysing proteins of disease it would be possible to link the excretion of disease biomarkers with future human health, potentially allowing the development of an early warning system for public health.

The primary challenge in analysing proteins for public health monitoring is one of scale. Attempts have been made before to analyse the health of a city by collection and analysis of thousands of individual urine samples [6]. Such invasive attempts to analyse public health are inefficient but have the advantage that the analytical techniques used to quantify protein biomarkers did not need to re-validated or adapted to work on this larger scale. WBE's greatest strength is that it replaces mass sample collection with collection of just a single representative wastewater sample. This approach has worked well for analysing a wide range of small molecular analytes [chapter four] , even those with very low excretion percentages like metabolites of heroin [7]. The difficulty for proteomic analysis of wastewater is that movement from urinary analysis to wastewater analysis brings about a big decrease in analyte concentration and a big increase in the complexity of the matrix. The concentration of current small molecule biomarkers in wastewater ranges from ng/L-g/L [8], depending on the substances. Whereas, the concentration of proteins in wastewater is currently unknown, although excretion varies from ng/L-mg/L in urine of health individuals [1]. The overall lower abundance of proteins is complicated by a lack of studies examining their stability in wastewater, meaning that actual protein concentrations may be even lower. However, this still puts the more abundant proteins into the same concentration range as other WBE suitable small molecule analytes. Solid phase extraction (SPE) has been used in both urinary proteomics [9; 10] as well as WBE [11-13] to increase sample concentration and reduce matrix interference. By applying the techniques of WBE to protein analysis it may be possible to analyse proteins in the same way as small molecules.

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is routinely used in both proteomics [9; 10; 14; 15] and WBE [11-13] for analyte quantification and identification. Additionally, by converting protein biomarkers into peptides, using an enzyme, it is possible to use the same range of LC and MS techniques for both proteomic [9; 16] and WBE [11; 13] applications. However, enzyme digestion necessitates a departure from established WBE protocols, which dictates that samples should be left unaltered and undergo sample preparation and SPE as soon as possible after collection. Additionally, a lack of understanding around the wastewater stability of proteins makes the addition of an enzyme digestion procedure an extra hurdle for analysts to overcome; although the stability of urinary biomarkers in the bladder suggests that proteins may be stable in wastewater for long periods [17].

The final considerations therefore surround the actual analysis of the protein/peptide biomarkers using LC-MS/MS. In literature the most commonly used type of chromatography is reverse phase liquid chromatography [14; 16; 18-20], often using the same type of silica based C_{18} end-capped columns used in WBE analysis [2; 3; 11; 21-23]. However, other chromatographic techniques are also used such as: ion-exchange chromatography (IEC) [24] or hydrophilic liquid chromatography (HILIC) [25; 26], particularly as second dimensions for analysis of peptides in complex mixtures [10; 27]. These other chromatographic methods seek to exploit the different combinations of polarity and hydrophobicity present within peptides, which is caused by their amino acid building blocks. HILIC has also been used within WBE but its usage is far less common and mostly restricted to very hydrophilic amphetamine-like drugs of abuse and drug metabolites [28-31]. For WBE the most common forms of MS detector are either quadrupole-time of flight (Q-ToF) or triple quadrupole (QQQ) type instruments, which are widely available in commercial and research labs worldwide. However, in proteomic analysis more advanced ion trap/triple quadrupole hybrid instruments are commonly used [14; 32-34], particularly in applications seeking to identify low abundance proteins in complex protein mixtures, although triple quadrupole type instruments are still used [16; 18]. Whilst proteins with a low predicted wastewater concentration could benefit from the additional sensitivity that more advance instrument can bring, for proteins with a similar abundance to small molecule biomarkers, the more routine QQQ and Q-ToF instrument may still be viable.

1.2 Literature analysis

The analysis of protein derived peptides, known as bottom-up proteomics, using mass spectrometry has several advantages compared to the analysis of intact proteins, known as top-down proteomics, including more developed analytical and quantification methodologies [35; 36]. The drawbacks of analysing proteins in this way are primarily an increase in the complexity of sample preparation and sample preparation time, as peptides are produced by incubating the protein with an enzyme for a period ranging from hours to days [37-39]. In addition to this, the majority of methods include extra pre-enzyme digestion steps aimed at increasing enzymatic efficiency by reducing protein structural complexity, usually through either disrupting hydrogen bonding, or reducing covalent bonds within the protein [40] as depicted in figure 1.

Figure 1. An overview of the peptide production process

Hydrogen bonding is important in determining the 3D structure of a protein, as the 2D amino acid sequence folds to allow for hydrophilic sections to interact with the aqueous intracellular environment whilst sheltering hydrophobic regions. Chaotropic reagents such as urea and guanidinium chloride (GuHCl), or surfactants including sodium dodecyl sulphate (SDS) or sodium deoxycholate (SDC) are commonly used within literature to disrupt hydrogen bonding in proteins [41]. However, this is not a requirement for bottom-up proteomics [37] and is almost always followed by the addition of other reagents before the addition of any enzyme. Additionally, many chaotropes will also interfere with the enzyme itself so their concentrations need to be considered carefully [40]. Likewise, urea can react with primary amines in amino acids in a process known as carbamylation [41]. Carbamylation is catalysed by both heat and time, which are both important for insuring efficient enzyme activity. Lastly, the use of surfactants can interfere with separation and ionisation in downstream LC-MS analysis [42] and so they should be removed, usually by either buffer exchange or by precipitation [43].

The strongest bonds holding a protein's 3D structure together are covalent disulphide bonds between cysteine amino acids. As the strongest intra-protein bond they play a key role in determining a protein's structure and their reduction increases the accessibility of the protein to the enzyme [40]. Generally, a relatively mild reducing agent is added to limit the reduction of other amino acids and the

most commonly used regent is dithiothreitol (DTT) [9]. A stronger reagent that can be used is tris (2-carboxyethyl) phosphine (TCEP), which has the advantage of being irreversibly oxidised after reduction, soluble in water and odourless compared to DTT [32; 44]. However, when either reagent is used in isolation the disulphide bond would reform, so after reduction an alkylating agent is generally added to prevent re-formation of the disulphide bond with the most common reagents being iodoacetamide (IAM) [32] and iodoacetic acid (IAA) [45].

The most important consideration in an enzymatic digestion is the choice of enzyme, as this directly determines the peptides that are produced. The most common protease reported in literature is trypsin [40], which cleaves the amide bond directly after arginine and lysine. Digestion times can vary widely from a few hours to days but the most common is the 16 hour digest (or overnight) which allows sufficient time for protein digestion [40; 41; 46]. If the enzyme digestion is quenched too soon, by addition of acid to the reaction, or if digest efficiency is low then there is a risk of producing peptides with missed cleavage sites, which complicates interpretation of the analysis.

Table 1. Summary of literature digestion methods highlighting the variety of methods reported in literature

Reference	Denaturant	Reducing agent	Alkylating agent	Enzyme	Digestion time	Comments
[47]	Urea and thiourea	DTT	IAM	Lys-C then Trypsin	3 hours then Overnight	A method was developed to produce peptides for non-targeted proteome analysis
[48]	SDS	TCEP	IAM	Trypsin	5 hours	Immunodepletion before digestion was used to improve detection of some proteins
[45]	GuHCl	DTT	IAA	Trypsin	8 hours	Assessed the effect of trypsin quality on digest efficiency

[42]	Rapigest, Invitrosol, PPS silent, Urea	TCEP	IAM	Trypsin	16 hours	This paper examined the use of different denaturants to optimise digestion
[49]	Urea, SDS	DTT	IAM	Trypsin, Chymotrypsin, Lys-C	Overnight	Three enzymes were compared. Trypsin and Lys-C were also used together to reduce the number of missed cleavage sites
[50]	Urea	DTT (x2)	IAM	Trypsin	14 hours	Extra DTT was added to quench excess IAM
[51]	n/a	DTT	IAM	Trypsin	Overnight	In-gel digestion followed by extraction and MALDI-ToF-ToF analysis
[52]	SDC	TCEP	IAM	Trypsin	16 hours	A method developed for analysing 67 biomarkers in plasma
[16]	Rapigest	n/a	n/a	Trypsin	4 hours	Proteins were precipitated from urine before digestion
[53]	Urea	DTT	IAM	Trypsin	Overnight	The paper examined the use of labelled intact proteins for peptide quantification

[39]	Rapigest	DTT (x2)	IAM	Trypsin (x2)	45-48 hours	Extra DTT was added to quench excess IAM, a second aliquot of trypsin was added after 24 hours, serum immunodepletion was carried out before digestion
[54]	Urea	DTT	IAM	Trypsin	Overnight	Serum immunodepletion was carried out before digestion
[32]	SDC	TCEP	IAM	Trypsin	16 hours	A method was developed for plasma protein analysis
[43]	Urea, SDS, SDC, Rapigest	DTT	IAM	Trypsin	5-7 hours	Surfactant removal by liquid-liquid extraction (LLE) and centrifugation were compared
[55]	Urea	DTT	IAM	Trypsin	16 hours	Samples were derivatised after digestion by sulphonating the C-terminal amino acid
[56]	GuHCl	DTT	IAA	Trypsin	22 hours	A method was developed for detection of medium abundance serum proteins

[57]	SDC	DTT	IAM	Trypsin	16 hours	Optimisation of SDC removal by acidification
[58]	SLA, Rapigest, SDC	DTT	IAA	Trypsin	16 hours	Sodium lauryl sulphate (SLA) was used as a novel surfactant
[37]	n/a	DTT (2x)	IAM	Trypsin	2 or 16 hours	Extra DTT was added to quench excess IAM
[59]	Urea	DTT	IAM	Trypsin (Bovine or Porcine)	8 hours	The effect of incubation at 37 ^{o}C and 48 ^{o}C and trypsin from different sources were compared
[60]	Urea, Rapigest, SDC	DTT	IAA	Trypsin, Lys-C	Overnight	Discussed the merits of LLE vs acidification for the removal of SDC compared to urea and Rapigest
[61]	Urea	DTT	IAM	Trypsin	16 hours	Compared method sensitivity and reproducibility using micro and nanoflow LC
[62]	SDC	TCEP then DTT	IAM	Trypsin	16 hours	Review of standardised protein kits, DTT was added to quench excess IAM

[19]	SDC	TCEP then DTT	IAM	Trypsin	16 hours	Assessment of the cost of MRM assay development, DTT was added to quench excess IAM
[41]	SDS, SDC, Urea, GuHCl, TFE	TCEP	IAM	Trypsin	0.5-23 hours	14 different combinations of heating, digestion temperature, chaotropes, surfactants and solvents were examined
[63]	Urea	TCEP	IAM	Trypsin	16 hours	Development of a high throughput, no LC method using online SPE-MS/MS and pre-digest enrichment with anti-peptide antibodies
[20]	Urea	DTT	IAM	Trypsin	3 hours	Different length LC gradients were used to try and reach LOQs similar to antibody-based methods, included immunodepletion before digestion
[64]	Urea	DTT	IAM	Trypsin	Overnight	A method of analysis using 2D-LC and MRM MS

[46]	Urea	DTT	IAM	Trypsin	0.5-16 hours	Paper focused on kinetics of adding isotopically labelled peptides for protein quantification
[9]	SDC	DTT	IAM	Trypsin	Overnight	Development of a single protein quantification method using synthetic urine
[27]	n/a	n/a	n/a	Trypsin	16 hours	High-abundance proteins were depleted using commercial kits
[38]	Urea	DTT (x2)	IAM	Trypsin (multiple sources)	2 or 16 hours	Extra DTT was added to quench excess IAM, Trypsin from 6 commercial sources was trialled to assess vendor variability
[65]	Urea and thiourea	DTT	IAM	Trypsin	Overnight	Proteins were extracted or concentrated using immunoaffinity columns prior to digestion

[66]	Urea – for cell lysis	TCEP	IAM	Trypsin	16 hours / overnight	Urea was used to help lyse cells and extract proteins before reduction and alkylation. Used antibodies to concentrate proteins before digestion
[33]	Urea, thiourea	Not specified	mTRAQ	Trypsin	16 hours	mTRAQ is a labelling agent and was used to isotopically label cysteines and for differential labelling of samples for MS analysis
[67]	n/a	n/a	n/a	Trypsin	Unspecified	In-gel digestion of pooled urine samples for gel electrophoresis analysis

From this snapshot a good starting point for a theoretical digestion protocol would be to use urea as a denaturant, followed by reduction with DTT, alkylation with IAM and finally a 16-hour trypsin digest. From the sources referenced above, this average method was only reported in 11% of sources, although if overnight digestion is considered the same as 16-hour digestion [66] then this rises to 19% of sources. An overnight or 16 hour digest is perhaps most commonly used due to its convenience, as well as ensuring sufficient time for peptide concentration to reach an end-point or steady state [41; 46]. Where the digestion was monitored by collecting samples over the duration of the digest, the concentration of peptides produced always reached its maximum before the end of the 16 hour [41] or 23 hour [46] digest, but the concentration of some peptides would then decrease as the digestion continued until quenching. Whilst the use of trypsin was fairly ubiquitous, other enzymes could be used including endoproteinase lys-C [47; 49] that cleaves after lysine, and chymotrypsin [49] that cleaved after tryptophan, tyrosine and phenylalanine. Trypsin was observed to be the best performing enzyme in a direct comparison of trypsin, chymotrypsin and lys-C [49] in terms of the

number of peptides produced without missing any cleavage sites. Despite an observed bias for missing lysine cleavages in trypsin digests, the use of lys-C followed by trypsin did not reduce the number of peptides with missed cleavages. Chymotrypsin was the worst performing enzyme compared in this study due to its slower rate of reaction, and the presence of peptides with missed or incorrect cleavages in the final solution.

The biggest variability in the literature is regarding the use of denaturants, which was reflected by several method development papers that assessed the effects of different chaotropes and surfactants on digest efficiency. Despite being the most commonly used, (47%) urea did not compare favourably to other reagents. This was particularly true when compared to surfactants, such as SDS, sodium deoxycholate (SDC), Rapigest (Waters), Invitrosol (Thermofisher) and PPS silent (Expedeon) that produced better digest efficiency than urea under identical conditions [41; 42] without the risk of carbamylation. Rapigest, Invitrosol and PPS silent are all commercially available, proprietary surfactants marketed for use in enzyme digestion. However, SDS, SDC and Rapigest needed to be removed before LC-MS/MS analysis to prevent interference [42], whilst Invitrosol and PPS silent were non-interfering surfactants and did not need removing. SDS was the hardest to remove in this regard with buffer exchange proving to be the best method for its removal [43], whilst Rapigest was the easiest as it is acid labile and could be made non-interfering by addition of acid after digestion. SDC was harder to remove than Rapigest but would precipitate under acidic conditions where it could be removed [41; 43] or alternatively it was removed via phase-transfer or liquid-liquid extraction (LLE) with ethyl acetate under acidic conditions [41; 43; 57]. As acid precipitation could cause unwanted peptide co-precipitation, LLE was reported as being more efficient at removing the surfactant without removing analyte [41; 43]. Where direct comparisons were possible SDC was reported as outperforming urea, Rapigest and SDS [41; 43; 57] and so it should be considered as a replacement for urea in enzyme digestion.

Another interesting technique was the use of organic solvents, such as methanol (MeOH), acetonitrile (MeCN) and isopropyl alcohol (IPA) to denature proteins, either used independently or in combination with surfactants [41; 42]. However, whilst a combination of MeCN and Rapigest was observed to give the best digest efficiency for analysis of cancer cell lysates [42], the use of MeOH was observed to decrease digest efficiency. Conversely, the use of MeOH and MeCN without surfactant was observed to increase digest efficiency for analysis of human plasma proteins [41] and, although not as much as when using SDC, they could be mixed together or with trifluoroethanol (TFE) to achieve comparable digest efficiencies. The use of common organic solvents to increase digest efficiency is intriguing due to its simplicity, but their benefits seem to be related to the proteins being studied with the potential to decrease digest efficiency as well as increase it. For this reason, when developing a new enzyme digestion method, surfactants are preferably to urea and other

chaotropes as they have been observed to only increase digest efficiency for a broad range of different proteins and matrices.

The final stage of all enzymatic digestion was the quenching of the digest with acid [49], usually using formic acid or TFA, and was generally followed by an extraction or similar process to prepare the sample for analysis. The most commonly extraction technique was solid-phase extraction (SPE) using either Oasis HLB (Waters) [9; 19; 41; 52; 62] or reverse-phase C_{18} [20; 43; 45; 50; 56; 58] stationary phases. Other methods were performed using molecular weight cut-off (MWCO) filters [37; 43; 46; 65] that allowed for protein concentration before digestion, or C_{18} spin filters that allowed for peptide concentration and surfactant removal after digestion [38]. The use of both kinds of filters simplified the digestion and extraction process but sample volumes were limited by the volume of the filter, whereas SPE is able to extract and concentration larger digestion volumes, which would be beneficial for analysing less abundant proteins.

1.3 Human proteins in wastewater

1.3.1 Human specific biomarkers

The use of WBE for understanding any public health question raises the issue of where the analyte originates. In studies of drugs of abuse or human pharmaceuticals the source of an analyte is expected to be consumption [68], disposal [69; 70] or metabolism [71]. When investigating the wastewater proteome, the source of a protein must be determined as human in origin if it is to be used for interpreting human health. Consequently, in bottom-up proteomics the focus must be on analysing human specific peptides originating from digestion of human proteins, as human and non-human proteins may share much of their amino acid sequences. In chapter four [1] seven potential WBE protein biomarkers were proposed: Prostate Specific Antigen (PSA), C-Reactive Protein (CRP), Interleukin-6 (IL-6), Interleukin-8 (IL-8), Podocin (PDC), Anterior Gradient Protein 2 (AGR2) and Uromodulin (URM). The peptides produced from digestion are dependent on the enzyme used to digest. Table 1 shows the human specific and non-specific peptides from an in-silico enzymatic digest using trypsin for each of the seven proteins listed above. In-silico digestion and monoisotopic peptide masses were performed using ExPASy online peptide mass programme. The search parameters left cysteines in their reduced form and assumed no missed cleavages. Peptides are presented in table 2 in a shortened format consisting of the first three amino acids of the sequence, a hyphen, and the last amino acid of the sequence, usually K or R. For example, the PSA peptide WTGGK is shortened to WTG-K. Human specificity was determined by searching for the sequence using Uniprot's online peptide search, treating leucine and isoleucine as the same to help identify isobaric peptides and only considering reviewed results.

Table 2. Specificity of tryptic peptides from biomarkers of disease, non-human specific peptides with asterisks (*) could be considered pseudo-specific, as discussed below the table.

Protein	Peptide (Molecular weight (Da))		Comments
	Human specific	Non-human specific	
Prostate specific antigen (PSA)	DTI-P (729.4) FML-R (796.4) LSE-K (1271.7) FLR-R (1870.9) LQC-K (2345.2) HSL-K (3492.7) STC-K (3965.9)	WTG-K† (547.3) VVH-R† (672.4) SVI-R* (728.5) IVG-K*† (1019.5) HSQ-R* (1406.7) AVC-R* (2229.2)	*= shared with *Macaca mulatta* and/or *Macaca fascicularis* † = shared with another human protein (KLK2)
C-reactive protein (CRP)	QTD-R (736.3) APL-K (866.6) ESD-K (1127.5) GYS-K (1135.6) QDN-K (1391.7) YEV-P (1819.9) AFT-R (1873.9) DIG-R (5109.5) GYT-R (6990.3)	AFV-K (707.4)	AFV-K is shared by *Mus musculus*, *Mesocricetus auraatus*, *Sus scrofa* and *Oryctolagus cuniculus*
Interleukin-6 (IL-6)	DVA-R (764.4) VPP-K (828.4) DGC-K (1775.7) NLD-K (1985.0)	ETC-K (593.2), AVQ-K* (763.4), SNM-K* (884.3), QPL-R* (916.5), EFL-R* (978.6), VLI-K* (987.6), FES-R* (1081.5), YIL-R* (1119.6), EAL-K (1324.7), LQA-R* (2208.1), IIT-R* (2212.2)	* = Shared with infraorder *Simiiformes* of the order primates, including macaques ETC-K and EAL-K are very non-specific
Interleukin-8 (IL-8)	None	LSD-R (546.3), CQC-K (593.3), EGA-R (740.4), ELC-K* (816.4), ENW-R (830.4), TYS-K* (1103.6), VIE-K* (1708.9)	* = shared with the family *Cercopithecidae* including macaques

Anterior gradient protein 2 (AGR2)	DTT-K (915.5), LYA-K (1940.0),	EIQ-K (516.3), YSN-R (538.3), DSR-K (601.3), ADI-R (631.3), VFA-K (706.4), LPQ-R (813.5), HLS-R (1267.6), IMF-R (1276.7), LAE-K (1995.1), GQG-K* (2200.0), TSN-K* (2511.3)	* = shared with *Pongo abelii* only
Podocin (PDC)	SIA-K (731.4), AGT-R (783.4), MIA-K (861.4), QAE-R (1170.5), AVQ-K (1264.7), APA-R (1340.7), VAL-K (1403.7), LQT-K (1666.9), DMF-R (1781.8), TQG-K (2119.1), GSG-K (2402.1), SSG-K (3632.9), YLH-R (3769.0)	VDL-R (501.3), IEI-K (501.3), DSP-L (561.2), LLA-R (608.4), VII-R (646.4), AAS-R (732.4), LGH-R (861.5), VVQ-R (921.5), SLT-R (1072.6), MAA-R (1626.9), MEN-K (1698.9), LPA-R (1788.9), GPG-K (1853.9)	All non-human specific peptides are shared with *Mus musculus* and *Rattus norvegius*
Uromodulin (URM)	TAL-R (1412.8), MAL-R (4231.0), LSP-R (4379.0)	VSL-K (502.3), SNN-R (546.3), GWY-R (580.3), DLN-K (601.3), CPH-R (612.3), GVQ-S* (660.3), SLG-K (665.3), DTS-R (677.3), FVG-R* (790.4), CSG-R* (797.3), WHC-K (803.3), FSV-R (913.4), SGS-R* (947.5), CKP-R (951.4), YFI-R (953.5), LEC-K* (979.4),	* = shared with *Pongo abelii* only

	TLD-R (981.5), VLN-R (981.6), DGP-R* (1017.5), VGG-R (1023.5), VFM-R* (1116.5), MAE-R (1117.6), DWV-R (1128.6), INF-K (1400.6), STE-R* (1678.7), QDF-R* (1669.9), DST-R* (1704.8), ACA-K* (2099.9), FAL-K* (2257.1), NET-R (2336.2), CNT-R* (2442.1), FAG-K* (2739.2), GDG-R* (3640.4), ACA-K* (4505.8), WCS-R* (7168.8)

All the protein biomarkers, except IL-8, had at least one human specific peptide biomarker. Fortunately, many of the IL-8 biomarkers are only shared with other primates, as is the case for many of non-specific peptides of other proteins, meaning they could still be used in countries without a large primate population; such as the UK. More problematic peptides are those shared with common European animals such as *Mus musculus* (house mouse), *Rattus norvegius* (brown rat) and *Canis lupus familiaris* (domestic dog), as these species may also contribute urine to wastewater, as well as those held in common with bacteria, fungi and yeasts that may colonise the inside of sewers or otherwise be discharged into them by natural or anthropogenic activity.

The selection of human specific peptides has not been a concern in previous clinical proteomic publications. As such, a variety of non-human specific peptides have become routinely used for analysis of the protein biomarkers listed above. For example, the analysis of URM via LC-MS/MS [72] used the peptides STE-R, shared with *Pongo abelli* (Sumatran orangutan), and DWV-R, shared with *Pongo abelli* and *Canis lupus familiaris*. Likewise the peptides IVG-K and LSE-K are ubiquitously used for analysing PSA [18; 73; 74] but IVG-K is shared by the related human kallikrein-related peptidase 2 protein and LSE-K has a single nucleotide polymorph (SNP) in humans causing the PSA protein to be expressed as LSE(I)-K, LSE(L)-K or a mixture of the two [75]. Podocin

and CRP meanwhile are largely analysed in bottom-up proteomics using human unique peptides, with separate studies of podocin using APA-R (Simon) and QEA-R [16] respectively.

1.3.2 Estimating human protein concentrations in wastewater

In WBE the last step after analysis is known as back-calculation [1; 76] and comprises all of the mathematical manipulation and interpretation required to take a result from a measurement of a sample in a vial through to a reliable estimation of the average amount consumed by a given population over a given time (usually 1000 inhabitants^{-1} day^{-1}) [76]. A more thorough accounting of how this process takes place can be found in chapter one. In brief, the concentration of analyte in a sample is multiplied to account for any increases in concentration that occurred from sample preparation, such as SPE. I.e. a 50 mL wastewater sample is concentrated down to 0.5 mL, so when back calculating the concentration in the sample is first multiplied by 0.1. Next, the concentration is multiplied by the daily wastewater flow (L day^{-1}) and then multiplied by 1000 divided by the population (1000 inh^{-1}) to normalise the results. This produces a value that can be compared with others taken in a different place and/or at a different time. For the purposes of estimating the protein concentration in wastewater, known as the load, this process can be reversed as shown below in equation 1 (eq. 1).

$$Wastewater\ load\ (g\ L^{-1}) = \frac{V_{urine} * C_{biomarker} * Population}{V_{wastewater}}\ eq.1$$

V_{urine} (L day^{-1}) is the volume of urine that an average person excretes each day and is multiplied by the average urinary biomarker concentration of a healthy individual, $C_{biomarker}$ (g L^{-1}), to calculate the amount of biomarker one healthy individual would excrete each day (g day^{-1}). This is then multiplied by the population served by the wastewater treatment works to calculate the total amount of biomarker excreted by the whole population each day. Finally, to account for dilution in wastewater caused by the presence of non-urinary water, such as rainfall, the result is divided by $V_{wastewater}$ (L day^{-1}). V_{urine} can be calculated using clinical estimates for a healthy individual [77], which suggest a healthy adult should excrete 1 ml kg^{-1} hr^{-1} of urine. Therefore, the average adult weight in the studied country, in this case the UK, is needed. The Office for National Statistics (ONS) [78] estimated in 2010 that the average UK adult male weighed 83.6 kg, whilst the average UK adult woman weighed 70.2 kg, for an average weight of 77 kg (assuming equal gender balance). V_U can be calculated using equation 2.

$$V_U = 1 * 77 * 24 * 10^{-3} = 1.848\ L\ day^{-1}\ eq.2$$

Using the healthy urinary biomarker concentrations presented in chapter four [1], the population of the chapter 1 UK study city [76] and the average wastewater volume for 2014-2018 from chapter 1 SI

table 2 (229,804,840 L day^{-1}) the wastewater load of each biomarker can be calculated. An example for C-reactive protein is shown below in equation 3 (eq. 3).

$$CRP\ load\ (g\ L^{-1}) = \frac{1.848 * 0.0012 * 886650}{229{,}804{,}840} = 8.5\ \mu g\ L^{-1}\ eq\ 3.$$

Uromodulin: 0.15 µg L^{-1}

Podocin: 28.9 ng L^{-1}

Interleukin-6: 11.4 pg L^{-1}

Interleukin-8: 49.9 pg L^{-1}

The difference between men and women in average, healthy, urinary concentrations of PSA meant each gender's contribution had to be calculated separately and then averaged. This was performed again assuming equal division of gender in the population, i.e. each gender's calculated used a population of 443,325 and calculated V_{urine} using the average weight of their gender.

PSA (Male): (V_{urine} = 2.006 L day^{-1}) 87.0 ng L^{-1}

PSA (Female): (V_{urine} = 1.685 L day^{-1}) 0.23 ng L^{-1}

PSA (Average): 43.6 ng L^{-1}

These estimated wastewater concentrations are subject to a number of assumptions, the biggest of which are that it assumes all of the population are adults and that all of the population is healthy. As such they should be used as a benchmark for assessing the analytical challenges of detecting and quantifying the biomarkers rather than for determining health interventions or as a measurement of population health. The concentration range of these biomarkers extends from pg-µg L^{-1}, which fits largely with the concentration range of other WBE analytes [8] although the interleukins would be just outside of this range.

2. Experimental

2.1 Analytical instrumentation and materials

The development of a successful digestion method required two interconnected aspects to be working alongside each other, firstly there needed to be a validated method for analysing the results of a prospective digestion method and secondly there needed to be a reproducible digestion method to create samples for analytical method development. This chicken and egg scenario required the use of an already validated LC-MS/MS method, and this was provided by the Chemical Characterisation and Analysis Facility (now the material and chemical characterisation facility (MC2)) of the University of Bath.

The first analytical method used an HPLC-Chip cube system that was directly coupled to an Agilent 6520 quadrupole-ToF (Q-ToF) mass spectrometer (Agilent Technologies, Santa Clara, CA) fitted with an electrospray ionisation (ESI) source and operated in positive ionisation mode. Liquid chromatography separation and sample enrichment was performed on the chip using a 40 nL enrichment column and 43 mm x 75 μm analytical column packed with 5 μm, Zorbax 300SB-C8 particles (G4240-63001, Agilent, Santa Clara, CA). An injection volume of 2 μL was used for samples with a total protein concentration 30nM, an injection volume of 1 μL was used for samples with a total protein concentration 60 nM and above this concentration either a 0.1 μL or 0.3 μL injection volume was used. Samples were first loaded onto the enrichment column at a rate of 4μL/min using the initial conditions shown below (Table 2). After 1 minute the samples were transferred onto the analytical column at a flow rate of 6 μL min^{-1} and the linear gradient was started. The solvents used were A: water with 0.1% formic acid (FA) and B: 90:10 acetonitrile: water, with 0.1% formic acid.

Table 3. Chip LC gradient with enrichment prior to analytical separation

Time (minutes)	% mobile phase A: H_2O + 0.1% FA	% mobile phase B: 90:10 MeCN: H2O + 0.1% FA
0.0	97	3
1.0	97	3
28.0	50	50
30.0	0	100
31.0	0	100
32.0	97	3

The MS ChipCube source was interfaced with an Agilent 1260/1200 series HPLC system consisting of a 1260 capillary pump, a 1200 Nano pump, a 1200 Micro WPS and a 1290 infinity thermostat (Agilent, Santa Clara, CA). The ChipCube source operated at a temperature of 365 ^{o}C, with a N_2

drying gas flow rate of 5 L min^{-1} and a capillary voltage of 2.1 kV. Data acquisition was carried out in a data dependant manner, with initial ToF only MS acquisition between 300-1700 m/z, with a scan rate of 4 spectra S^{-1}. Once a precursor was detected at or above an intensity threshold of 2000 counts the spectrometry conditions changed to MS/MS mode, with a collision energy proportional to the detected m/z, a scan rate of 3 spectra S^{-1} and data acquisition between the range of 50-1700 m/z.

Peptides were identified using Matrix science's Mascot peptide database, which takes imported MS/MS data and information about the digest, such as the enzyme used, and generates a list of detected peptides and the proteins that they could belong to. An added benefit was that the software identified peptide sequences that were unique to a particular protein and which were shared between related species or related proteins. The software was set up to allow for up to 2 missed cleavages and both methionine oxidation and cysteine labelling with iodoacetamide. Where urea was used in digests carbamylation was also selected as a possible peptide modification.

All other experiments were performed using a Waters Acquity UPLC coupled to a Waters Xevo TQD triple quadrupole instrument with the following MS instrument conditions: Capillary voltage: 2.5 kV, Source temperature: 150.0 ^{o}C, Cone gas flow: 100.0 mL min^{-1}. The HILIC method used 100% H_2O with 0.1% formic acid as mobile phase A and 100% MeCN with 0.1% formic acid as mobile phase B. The flow rate used was 0.3 mL min^{-1} and a mobile phase gradient was developed as detailed below in table 4. HILIC separations were performed using a Waters Acquity UPLC BEH column, 1.7 µm, 1.0 x 100 mm.

Table 4. HILIC method gradient

Time (minutes)	% mobile phase A: H_2O + 0.1% FA	% mobile phase B: MeCN + 0.1% FA
0.0	5	95
2.0	5	95
22.0	50	50
27.0	50	50
27.1	5	95
40.0	5	95

Acetonitrile (MS grade), methanol (MS grade) and ethyl acetate (>95%) were purchased from VWR. Formic acid (>98%), ammonium bicarbonate (>99.5%), hydrochloric acid (≥32% purity), sodium deoxycholate (>98%), dithiothreitol (>98%), iodoacetamide (>99%), trypsin (porcine, di-methylated) and Whatman GF/F filter paper were purchased from Sigma Aldrich, UK. Prostate specific antigen

(Human), Bovine serum albumin (Fraction V), C-reactive protein (Human), Interleukin-6 (Human) and Interleukin-8 (Human) were all purchased from Merck. Oasis HLB SPE cartridges (3 CC, 60 mg) and 300 µL PPE vials were purchased from Waters UK. 4 mm, 0.2 µM Titan^3 PVDF syringe filters were purchased from Thermo scientific.

2.2 Enzyme digestion development

The goal of digest development was to create a method that reproducibly produced protein unique peptides from several different biomarkers, and that could be adapted to work with more complex matrices like wastewater. The proteins used to develop the digest were prostate specific antigen (PSA) and bovine serum albumin (BSA). PSA is a well-studied biomarker of human health that is excreted in urine and used as a diagnostic biomarker for prostate cancer in men, as discussed in chapter four [1]. BSA was included as it is a relatively cheap protein that could be used as an internal control in future digests with real samples to ensure that the digest procedure worked and to assess digest efficiency. Samples were extracted using Oasis HLB cartridges and eluted and reconstituted to a specified final protein concentration. Before extraction cartridges were conditioned using 2 mL of MeOH followed by 2 mL of H_2O with 0.1% v/v formic acid. Oasis HLB cartridges were selected as they have a broad selective that would allow for the simultaneous extraction of as many peptides as possible. After loading samples were eluted in 4 mL of 80:20 MeOH: H2O with 0.1% formic acid and placed in a water bath at 40 ^oC before evaporating to dryness under a steady stream of N_2. Samples were then reconstituted to a specified final volume in a specified mobile phase. Figure 2 (below 2.2.4) shows a summary of all the digest conditions trialled.

2.2.1 Enzyme only method

The enzyme only digest protocol was performed in duplicate and had 10 pmoles of BSA and PSA in individual samples 1 mL containing 100 mM NH_4HCO_3 buffer at pH 8.4. Trypsin was then added, and the proteins were left to digest for 16 hours before quenching with 0.5% v/v formic acid. Samples were extracted using Oasis HLB cartridges and loaded under gravity for 5 minutes before being washed with 3 mL of 0.1% v/v formic acid solution. After drying, samples were eluted and reconstituted as described above (2.2) to a final protein concentration of 30 nM in 300 µL of 95:5 H_2O: MeOH for analysis via the Chip cube method.

The method was repeated in duplicated with a greater initial concentration of both PSA and BSA to give a final protein concentration of 303 nM and 606 nM respectively.

2.2.2 No surfactant methodology

A more comprehensive methodology was then developed using DTT and IAM solutions to reduce and alkylate the two proteins prior to digestion with trypsin, with the goal of increasing digest efficiency

by increasing trypsin's ability to access cleavage sites in the proteins. This was expected to be particularly beneficial for BSA which contains seventeen disulphide bonds, whereas PSA contains only five. The final protein concentration of BSA and PSA was reduced back to 30 nM in a final volume of 300 µL.

2.2.3 Use of sodium deoxycholate (SDC)

To assess the benefits of using a surfactant to denature the proteins before digestion 100 µL of 7% (m/v) aqueous sodium deoxycholate (SDC) solution was added to samples containing either BSA or PSA before addition of DTT. As a denaturant SDC was expected to increase digest efficiency and was shown to be more beneficial than urea. However, SDC required removal before analysis, which was performed by precipitating SDC when quenching the digest, using 0.5% v/v formic acid, with the rest of the digest was carried out as detailed in the no surfactant methodology (2.2.2) until after the digest was quenched. After quenching the samples were left to precipitate for 15 minutes and then centrifuged at 1000 RPM for 1 minute to help pellet the precipitated SDC and the supernatant was then loaded onto cartridge as normal. SPE and sample preparation then continued as before with samples reconstituted to a volume of 300 µL giving a final protein concentration of 30 nM.

An alternative SDC removal method using liquid-liquid extraction with ethyl acetate was also trialled to compare against acid precipitation. The digest was carried out as above but before the digest was quenched 1 mL of ethyl acetate (1:1 v/v) was added to each sample and 0.5% v/v formic acid was then added to quench the digest. The digests were then shaken, and the two phases were allowed to separate before the ethyl acetate was removed by pipetting. This process was repeated twice more, without addition of more formic acid, before the aqueous fraction was loaded onto cartridge as before. SPE and sample preparation then continued as before with samples reconstituted to a volume of 300 µL giving a final protein concentration of 30 nM.

2.2.4 Use of urea

Whilst less efficient and more likely to carbamylate peptides urea was trialled as an alternative to SDC, as urea had the advantage of being removed by SPE without the need for extra sample preparation. The digest procedure was carried out as described in 2.2.2 but before addition of DTT 100 µL of 6 M urea was added. From addition of DTT, IAM and additional buffer solution the concentration of urea was reduced to <0.6 M to prevent urea from interfering with trypsin activity. SPE and sample preparation were carried out as before with samples reconstituted to a volume of 300 µL giving a final protein concentration of 30 nM.

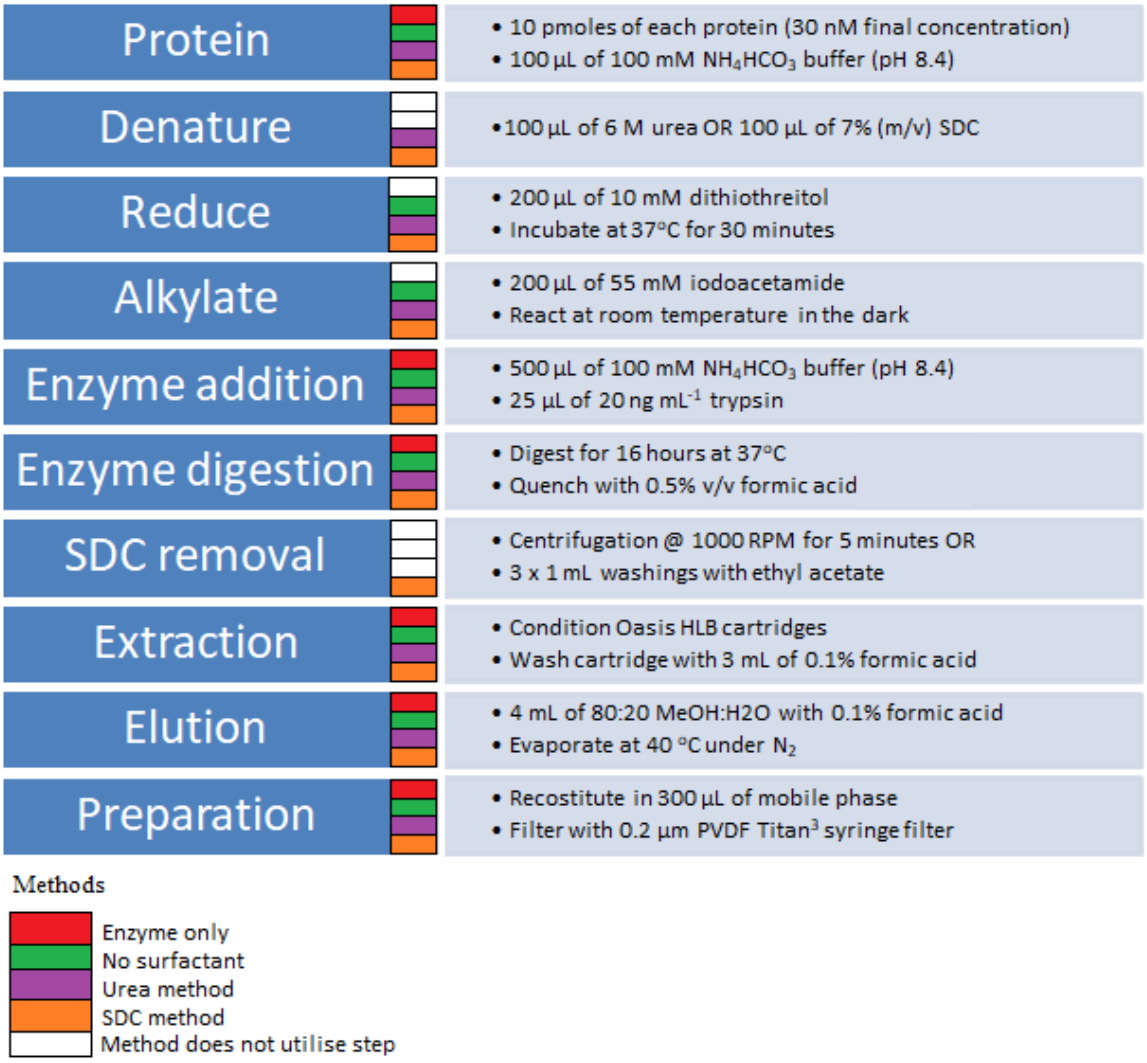


Figure 2. Summary of digestion experiments with colour coding to show the steps that were used by each method.

2.3 Assessing digest robustness

2.3.1 Co-digestion of BSA and PSA

Further work was then carried out using the best performing digest from section 2.2 to assess the impact on digest efficiency of combing multiple proteins into one digest, in this instance BSA and PSA, each at an individual concentration of 30 nM.

2.3.2 Digestion of new biomarkers

New protein biomarkers were selected and digested alongside BSA, at an increased individual concentration of 500 nM, using the method described above. The final concentration was increased so that they could more easily be used with the HILIC-MS method described in section 2.4. The selected biomarkers were C-reactive protein (CRP), interleukin-6 (IL-6) and interleukin-8 (IL-8), all of which are biomarkers of inflammation, with CRP also being a biomarker of cardiovascular disease and the two interleukins as biomarkers of urinary tract infection. The proteins also show a large discrepancy

in mass range and were selected to check if protein size impacted digest efficiency. IL-8 has a mass of 8.4 kDa and was the small protein examined here, IL-6, CRP and PSA all have relatively similar masses (21 kDa, 24 kDa and 26 kDa respectively), whilst BSA is the largest protein with a mass of 66 kDa.

2.4 Quantotypic peptides

The peptides produced from all the digest experiments carried out in 2.2 and 2.3 were recorded and compared to the list of human unique peptides contained in table 2 in order to identify quantotypic peptides for each of the biomarkers. For this purpose, peptides with missed cleavages were also considered quantotypic but were only used to determine sequence coverage if the fully cleaved peptide was not detected. For example: DTI-P is a human unique peptide of PSA, WIK-P is a related peptide where the bond between K and D was not cleaved. As such WIK-P is still a human unique peptide of PSA but only counted towards sequence coverage if DTI-P was not detected.

2.5 HILIC-MS method development

Method development focused on the use of HILIC for the separation of quantotypic peptides identified in the previous sections (2.2 and 2.3). In section 1.3 a complete range of tryptic peptides for several potential biomarkers was produced from their in-silico digestion via trypsin. These peptides encompass a wide range of physical properties resulting from the differences in their constituent amino acids. HILIC was chosen instead of the more commonly utilised place reverse phase chromatography to take advantage of the fact that almost all digested peptides would have a charged, hydrophilic C-terminal peptide giving the peptide some HILIC retention. Additionally, the presence of polar amino acid side chains allowed for a secondary mode of interaction with the stationary phase.

Binary two proteins digests containing 5 µM of bovine serum albumin (BSA) and either CRP or PSA were prepared using the method developed in section 2.2. PSA and CRP were selected above other biomarkers discussed in section 1.3 due to their relatively high urinary concentration and clear disease-biomarker relationships, i.e. a disease state progresses the amount of biomarker excreted increases. Samples for HILIC analysis were reconstituted in 50:30:20 (% v/v) MeCN: MeOH: H2O with 0.1% formic acid. This diluent was used for reconstitution of HILIC samples after reconstitution in mobile phase (95:5 (% v/v) MeCN: H2O with 0.1% formic acid) was unable to fully dissolve the sample.

The use of a triple quadrupole instrument necessitated the selection of MRM transitions for the peptides. MRM transitions were selected by infusing 5 µM digests of each biomarker into the instrument at a flow rate of 20 µL min^{-1}. Ionisation conditions were then altered to determine optimal conditions for the ionisation of the precursor peptides at a collision energy of 0 V in full scan MS

mode. Next the collision energy was increased so that the precursor peak intensity was reduced by half and peptide daughter ions were then identified. The collision energy was then altered to find the optimal energy to produce the greatest daughter ion peak intensity possible. The three most intense peptides and their most intense transitions were recorded and confirmed by injection and analysis of the remaining 5 µM digests using the HILIC method outlined above (section 2.1). The HILIC method was kept deliberately broad by starting at a low percentage of mobile phase A and slowly increasing to 50% in order to provide some separation for all the potential biomarkers. The flow rate selected was the fastest possible whilst keeping the maximum column pressure below 80% of its maximum operating pressure, which occurred at a 50:50 mixture of the two mobile phases.

Full instrument and method validation were not possible at this stage of development, so a preliminary method performance experiment was performed to examine the instrument's response to BSA and to gain an understanding of digest intraday reproducibility. Method performance was performed using BSA only digests at a range of concentrations. BSA linearity was determined for each of three BSA peptides, identified as quantotypic in sections 2.2 and 2.3, using a twelve-point calibration curve ranging from an initial BSA concentration of 0-1000 µg mL^{-1}. The calibration curve was created by spiking in differing initial amounts of a 1 mg mL^{-1} BSA solution into digests prepared according to the best performing method developed in section 2.2. The final concentrations selected were 0, 1, 2.5, 5, 10, 18, 32, 63, 125, 250, 250, 500 and 1000 µg mL^{-1}. Each sample was analysed twice using the HILIC method developed above and integrated using MassLynx (V4.1) with analyte peak areas used to generate a calibration curve. The linearity of this calibration curve was a measure both of analyte response and digest reproducibility. Accuracy was determined by comparing the concentration of each sample, as determined by the calibration curve, to the known initial BSA concentration (x) using equation two (eq. 2).

$$Accuracy\ (\%) = \frac{Theoretical\ BSA\ concentration_x}{Measured\ BSA\ concentration_x} * 100\ eq.2$$

Reproducibility was determined by injecting the same sample twice and comparing the difference in integrated peptide areas. Reproducibility was calculated as % RSD using equation three (eq. 3).

$$Precision\ (\%\ RSD) = \frac{\sigma\ Peak\ areas_{X_1X_2}}{Average(Peak\ area_{x1}, Peak\ area_{x2})} * 100\ eq.3$$

The goal of this work was to establish an effective linear range for BSA so that it could be considered as a potential marker for determination of digest efficiency in real samples, via the addition method [79].

Limits of detection and quantification were calculated using the slope (m) and standard deviation of the intercept (σ_C) of the linear concentration line of best fit, rather than the more traditional signal to noise approach. The standard deviation of the intercept was calculated using Microsoft Excel's regression function and LOD and LOQ were calculated using equation three (eq. 3) where x = 3.3 for LOD and x = 10 for LOQ. N was the number of points in the calibration.

$$LOD\ or\ LOQ\ = x * \frac{\sigma_C * \sqrt{N}}{m}\ eq.3$$

2.6 Analysis of wastewater samples

From predicted wastewater concentrations of PSA and CRP (section 1.3) it was possible to estimate how much wastewater would be required to achieve a protein concentration of 500 nM. For PSA the required volume was several litres and so not currently feasible but for CRP this was equivalent to 300 mL of influent wastewater. Whilst more reasonable this is still a larger volume than was used for most WBE applications, so initially a volume of 100 mL was used. 100 mL was selected as a starting volume because the estimate wastewater concentrations for both CRP and PSA assumed a 100% healthy population, which suggests that their actual wastewater concentration may be higher. Influent wastewater was collected from a treatment works in the South-West of the UK by grab sampling. As the reagents used for protein reduction and alkylation were already added in excess the volumes used were unchanged, as was the amount of trypsin added. Before digests were loaded on SPE cartridges, as discussed in section 2.2, they were first filtered through 0.2 µM Whatman GF/F filter paper under vacuum. The digest efficiency of trypsin is known to be determined by pH, so the pH of wastewater samples was measured after addition of each reagent and before and after acidification to ensure pH stability. Initial digests using wastewater compared three sets of conditions, which were prepared and analysed in duplicate, as follows:

1) A standard digest with 500 nM each of BSA, CRP and PSA in buffer
2) A 100 mL of wastewater digest with 500 nM each of BSA, CRP and PSA added
3) A 100 mL wastewater digest with 500 nM of BSA added

Each of the three digests tested a specific parameter of the methodology. Firstly, if the digestion and reagents were working (digest one), if proteins could be detected when spiked into wastewater (digest two) and if proteins endogenous to wastewater could be detected (digest three). BSA was added to all digests as a digestion control, i.e. if BSA peptides were present then the digestion worked.

Follow up digests tried adding different amounts of protein to wastewater digests to allow for better detection of analytes. This time four digests were prepared:

1) A buffer digest containing 500 nM each of BSA, CRP and PSA

2) A 100 mL wastewater digest with 500 nM of BSA added
3) A 100 mL wastewater digest with 500 nM each of BSA, CRP and PSA added
4) A 100 mL wastewater digest with 5 µM each of BSA, CRP and PSA added

In the same vain three more digests were prepared to examine the effects of adding more trypsin to digests, 1 mL of 20 ng mL^{-1} trypsin instead of 25 µL of 20 ng mL^{-1} trypsin and increasing the amount of wastewater used for the digests from 100 mL to 500 mL. As before three digests were prepared, but this time no buffer digest was prepared and the only protein added was BSA, which was added to a final concentration of 500 nM in each digest.

1) 100 mL of wastewater with 500 nM of BSA, digested with 25 µL of 20 ng mL^{-1} trypsin
2) 100 mL of wastewater with 500 nM of BSA, digested with 1 mL of 20 ng mL^{-1} trypsin
3) 500 mL of wastewater with 500 nM of BSA, digested with 25 µL of 20 ng mL^{-1} trypsin

The purpose of each of the three sets of digests was to examine the feasibility of using wastewater as a source of proteins of human health and to investigate the effects of different conditions on the developed digest methodology (section 2.2) to convert proteins of disease into their respective quantotypic peptides.

3. Results

3.1 Enzyme digestion development

Section 2.2 trialled a number of different digest methodologies with varying complexities as summarised in figure 2. All detected peptides were detected as the $[M+2H]^{2+}$ ion unless otherwise specified.

3.1.1 Enzyme only digests

The result of using only trypsin was that no BSA peptides were detected, whilst only one peptide of PSA was detected at low intensity (SI figure 1). As the final digest concentration (30 nM) was relatively close to the instrument's detection limit of 10 nM the final concentration of each biomarker was increased to 303 nM for PSA and 606 nM for BSA. This increased concentration resulted in better digest efficiency with more peptides detected for each biomarker (SI figures 2-5). Sequence coverage is a measure of how many peptides were produced relative to the number of peptides produced from an in-silico digestion of the protein and is a good way of assessing digest efficiency. The high concentration, trypsin only method managed to achieve an average BSA sequence coverage of 46.7% or 28.5 peptides, and an average PSA sequence coverage of 33.6% or 4 peptides (SI figures 2-5). This was an encouraging start but the concentration of proteins required to get this sequence coverage is very high when compared to urinary concentrations in healthy men (525 ng/L or 20.19 pM) [80]. The PSA peptides detected were SVI-R, WIK-P, LSE-K and HSQ-R, where WIK-P is the incomplete digest form of the DTI-P peptide reported in table 2, resulting from a missed cleavage between the K and D amino acids. Likewise, KWI-P was also detected. KWI-P, WIK-P and DTI-P were considered equivalent for determining sequence coverage and are all human unique peptides of PSA. KWI-P was detected as both $[M+2H]^{2+}$ and $[M+3H]^{3+}$ ions. LSE-K is also human unique peptide whilst SVI-R and HSQ-R are pseudo-unique for the purpose of wastewater analysis in the UK, as they are shared with primate species that should not be present in the UK.

3.1.2 No surfactant digests

A more comprehensive methodology was then developed using DTT and IAM solutions to reduce and alkylate the two proteins prior to digestion with trypsin, with the goal of increasing digest efficiency by increasing trypsin's ability to access cleavage sites in the proteins. The final concentration of BSA and PSA was reduced back to 30 nM for comparison with the initial trypsin only digest and for a better comparison of digest efficiency between BSA and PSA. For BSA the average sequence coverage was 86.7% with an average of 52 peptides detected, and for PSA the average sequence coverage was 40.3% with 6 peptides detected (SI figures 6-9). This was a marked improvement in digest efficiency and showed that reduction and alkylation of disulphide bonds was very important for ensuring high

digest efficiency. The PSA peptides detected included SVI-R, WIK-P, LSE and HSQ-R, which were also detected in the high concentration enzyme only digest, as well as FM*L-R and IVG-K. IVG-K is shared with human kallikrein protein 2 and is therefore not human PSA unique. FM*L-R is the methionine oxidised form of FML-R, which is a human PSA unique peptide and an example of a post-translation modification (PTM).

3.1.3 SDC containing digests

SDC was shown to be more beneficial than urea but required removal before analysis, which was performed by either precipitation SDC when quenching the digest using 0.5% v/v formic acid or liquid-liquid extraction (LLE) using ethyl acetate. After quenching with acid and the visible precipitation of SDC a clear, colourless gel formed that could only be re-dissolved by reducing the pH below pH 2, which was below the recommended pH for extraction using Oasis HLB SPE cartridges. If the gel was dissolved and then the pH increased to between pH 2 and pH 4 then the gel reformed. If the samples were loaded immediately after quenching, without waiting for SDC to precipitate, then this gel formed in the SPE cartridge and prevented analysis of the digest. A more pH tolerant stationary phase material could have been selected, such as a cation exchanging phases, but this would restrict the selectivity of the stationary phase and could have unfavourably biased the types of peptides that were detected.

With the LLE method of extraction no precipitate was visible during this process but there was also no gel formation and the samples could be extracted and eluted as normal. However, the final digests only contained peptides originating from porcine trypsin above LOQ, no peptides of BSA or PSA were detected.

3.1.4 Urea contain digests

Urea was used as an alternative to SDC, which had the advantage of being removed by SPE without the need for extra sample preparation. The concentration of urea was reduced to <0.6 M before addition of trypsin due to the addition of other reagents and buffer and should not have interfered with trypsin activity. However, this still led to no peptides of BSA or PSA being detected at a final concentration of 30 nM, showing it to be worse than the previous methods without any denaturants. For these digests the Mascot search parameters were expanded to include carbamylation as a potential peptide modification.

3.2 Assessing digest robustness

As none of the commonly used denaturants seemed to benefit the digest they were removed, and the digest described in sections 2.2 and 3.1.2 (no surfactant digest) was used for assessing digest robustness.

3.2.1 Co-digestion of BSA and PSA

The co-digestion of BSA and PSA was performed using the method described above with a protein concentration of 30 nM for each protein. (SI figures 10 and 11) Overall this caused a reduction in total sequence coverage for both proteins with 64.7% sequence coverage for BSA with 13 unique peptides, whilst PSA had an average of 26.9% sequence coverage with 4 unique peptides. Whilst disappointing this reduction in unique peptide coverage was still acceptable as the detection of any of these peptides indicated that the protein was present in the digest, and for both proteins this reduction represented the loss of only two unique peptide sequences. For PSA the four detected peptides were SVI-R, IVG-K, WIK-P and LSE-K representing one pseudo-unique, one non unique and two unique peptides of PSA respectively. This was particularly encouraging as SVI-R, IVG-K and LSE-K are the most commonly reported peptides of PSA in bottom up digests in literature.

3.2.2 Digestion of new biomarkers

New protein biomarkers were then selected and digested alongside BSA, at an increased individual concentration of 500 nM, using the method described above. The selected biomarkers were C-reactive protein (CRP), interleukin-6 (IL-6) and interleukin-8 (IL-8). The summary for all biomarker digests is shown below in table 5. CRP and IL-8 were injected twice at different injection volumes (0.1 µL or 0.3 µL) to try and maximise the number of peptides detected. IL-6 was not reinjected as eight peptides were determined to be sufficient.

Table 5. BSA + biomarker digest sequence coverages and digest efficiency as determined by mascot

Biomarker + BSA digests	Final concentration (nM)	Injection volume (µL)	Biomarker sequence coverage			BSA sequence coverage		
			%	# of peptides	# of peptides with missed cleavages	%	# of peptides	# of peptides with missed cleavages
BSA only	500	0.1	n/a	n/a	n/a	70.4	43	8
CRP	500	0.1	65.9	5	1	63.9	39	3
	500	0.3	94.2	6	0	91.7	56	3
IL-6	500	0.1	58.7	8	0	62.3	38	4
IL-8	500	0.1	39.8	4	0	50.8	31	2
	500	0.3	49.7	4	0	81.9	50	3

There were five CRP peptides detected in the 0.1 µL injection volume analysis (SI figure 12): AFV-K, ESD-K, GYS-K, RQD-K and YEV-P. AFV-K and an alternate form with a missed cleave, KAF-K, were the only human non-unique peptides detected. In addition to YEV-P the missed cleavage form ALK-P $[M+3H]^{3+}$ was also detected. RQD-K is the missed cleavage form of the unique peptide QDN-K. In total seven peptides were detected. By increasing the injection volume to 0.3 µL the following peptides were detected (SI figure 13) in addition to those reported in the 0.1 µL analysis: QDN-K, APL-K and ESD-K, as well as missed cleavage forms of several peptides. In total eleven peptides were detected.

With a final injection volume of 0.1 µL there were eight IL-6 peptides detected (SI figure 14): EFL-R, VLI-Q, YIL-R, EAL-K, DGC-K, NLD-K, LQA-R and IIT-R. NLD-K and LQA-R were the only human unique peptides detected, whilst EAL-K is non-specific and all others are pseudo-specific; being shared only with primates that are not expected to be found in the UK. Several missed cleavage forms of peptides were also detected so that in total, thirteen peptides were detected.

The first analysis of the IL-8+BSA digest with an injection volume of 0.1 µL contained only four peptides (SI figure 15): ENW-R, ELC-K, TYS-K and VIE-K, all of which are pseudo-unique as they are only shared with non-UK native primates. It is important to note that TYS-K and VIE-K were detected as $[M+3H]^{3+}$ ions. The second digest (SI figure 16) did not lead to anymore peptides being detected, although $[M+2H]^{2+}$ and $[M+3H]^{3+}$ ions of TYS-K and VIE-K peptides were detected, as well as a missed cleavage form of ELC-K, LSD-K.

Unique peptides of BSA have not been discussed as its role in the digests is to act as a control for digest efficiency. Instead what was needed was to identify peptides that were produced reproducibly regardless of their uniqueness. In total BSA was included in a total of twelve analysed digests from experiments 2.2.1-2.2.4 and 38 peptides were detected in at least half the digests. Of these 38 peptides fourteen were detected in ten or more digests: LVT-K, AEF-K, LVV-A, QNC-K , LVN-K, YIC-K, TCV-K, LGE-R, EYE-K, DDP-K, DAF-R, LKP-K, RPC-K and GLV-K. Additionally, LFT-K was detected in eight digests.

Overall, the final digestion method showed robustness in producing a range of peptides for each of the assessed biomarkers. Size did not appear to have a significant impact on digest efficiency, as assessed by percent sequence coverage. However, BSA sequence coverage across these digests was observed to fluctuate. The BSA+CRP, BSA+IL-6 and BSA+IL-8 digests were all performed at the same time and whilst the number of BSA peptides and the number of missed cleavages did vary between digests there were fourteen peptides, which corresponds to 21.3% sequence coverage, that were detected in every one of these digests. These peptides can be described as quantotypic, meaning they are produced reproducibly and can be thought of as being typical of that protein. The identification of quantotypic peptides is important for protein detection and quantification in real samples. At this stage the goal was only to show that the digest protocol could successfully digest a range of different proteins with a range of different masses and assessing how digest efficiency was impacted by having them all together was thought of as more application than development.

The analytical method used here was beneficial for achieving high sensitivity without the need for large sample volumes. However, the chip LC-MS/MS system used is relatively niche in proteomics [50; 61] compared to more common standard flow chromatography. The goal of the project was to create a digestion procedure that could be used to prepare protein samples for analysis using the same instrumentation and methodologies as those currently used to analyse other WBE biomarkers. As such this required a move away from the Chip-LC method to more conventional UHPLC. Additionally, whilst using SPE to concentrate peptides was not really required for these samples it would need when working with more dilute protein solutions and more complex matrices, such as wastewater.

3.3 HILIC-MS method development

5 µM digests of BSA+CRP and BSA+PSA were prepared in accordance with the procedure detailed in 2.2 and used to tune the Xevo TQD MS and identify peptide ionisation conditions and MRM transitions. During infusion of analytes it became necessary to switch to a combined flow where mobile phase was used to assist analyte ionisation. 100% of mobile phase B (100% MeCN with 0.1% (v/v) formic acid) was flowed into the MS at a rate of 0.1 mL min^{-1}. Into this flow of mobile phase analytes were infused from the MS module at a rate of 20 µL min^{-1}. Using these conditions, the most abundant peptides of BSA, PSA and CRP were identified. For CRP the peptides $[YEV\text{-}P]^{2+}$ and $[ALK\text{-}P]^{3+}$ were as abundant as each other and so both were included as they were both related to the same CRP unique peptide. Fragmentation of the parent peptides then lead to the detection of at least two daughter ions for each parent, with the most intense two selected for future use. The MRM conditions used are shown below (Table 6) and source conditions are listed below:

Capillary voltage: 2.5 kV, Source temperature: 150.0 ^{o}C, Cone gas flow: 100.0 mL/min,

Table 6. Peptide MRM conditions for the triple quadrupole method

Protein	Peptide	MRM 1	CV (V) / CE (V)	MRM 2	CV (V) / CE (V)
BSA	LGE-R	740.5 > 171.1	50 / 45	740.5 > 300.1	50 / 30
	LVN-K	582.3 > 213.2	35 / 20	582.3 > 951.5	35 / 21
	LVT-K	395.2 > 213.2	30 / 11	395.2 > 74.1	30 / 45
CRP	YEV-P	911.0 > 622.8	60 / 34	911.0 > 703.9	60 / 34
	ALK-P	711.7 > 715.4	35 / 35	711.7 > 559.3	35 / 35
	GYS-K	568.8 > 124.6	30 / 18	568.8 > 221.1	30 / 12
	APL-K	434.3 > 398.8	40 / 5	434.3 > 147.1	40 / 5
PSA	LSE-K	636.8 > 943.5	40 / 25	636.8 > 646.4	40 / 25
	IVG-K	539.2 > 213.0	25 / 20	539.2 > 283.5	25 / 12
	SVI-R	379.2 > 458.3	25 / 11	379.2 > 232.14	25 / 11

Peptide retention times are shown below in table 7 and chromatograms from injection of the tuned samples are shown in figures 6-13. The CRP peptide GYS-K had a very unusual peak pattern of five peaks, which was not observed in the chip-LC experiments. At this stage no reason could be suggested for why this occurred, other than an insufficient number of points per peak (ppp) causing a broad peak to appear chopped into several smaller peaks. However, the method had >30 ppp, which was regarded as sufficient for quantification previously on this instrument (Chapter 3, [81]) and no other peptide was affected by this apparent peak chopping.

Table 7. Average retention times of tuned peptides using average peak top retention time for each daughter (MRM) ion

Protein	Peptide	Average retention time (min) ± deviation (min)
BSA	LGE-R	9.31 ± 0.00
	LVN-K	9.46 ± 0.02
	LVT-K	8.96 ± 0.05
CRP	YEV-K	8.02 ± 0.02
	ALK-P	8.00 ± 0.00
	GYS-K	14.43 ± 0.02
		16.80 ± 0.02
		18.80± 0.02
		20.57 ± 0.02
		22.89 ± 0.08
	APL-K	8.13 ± 0.01
PSA	LSE-K	10.38 ± 0.07
	IVG-K	9.14 ± 0.00
	SVI-R	7.97 ± 0.06

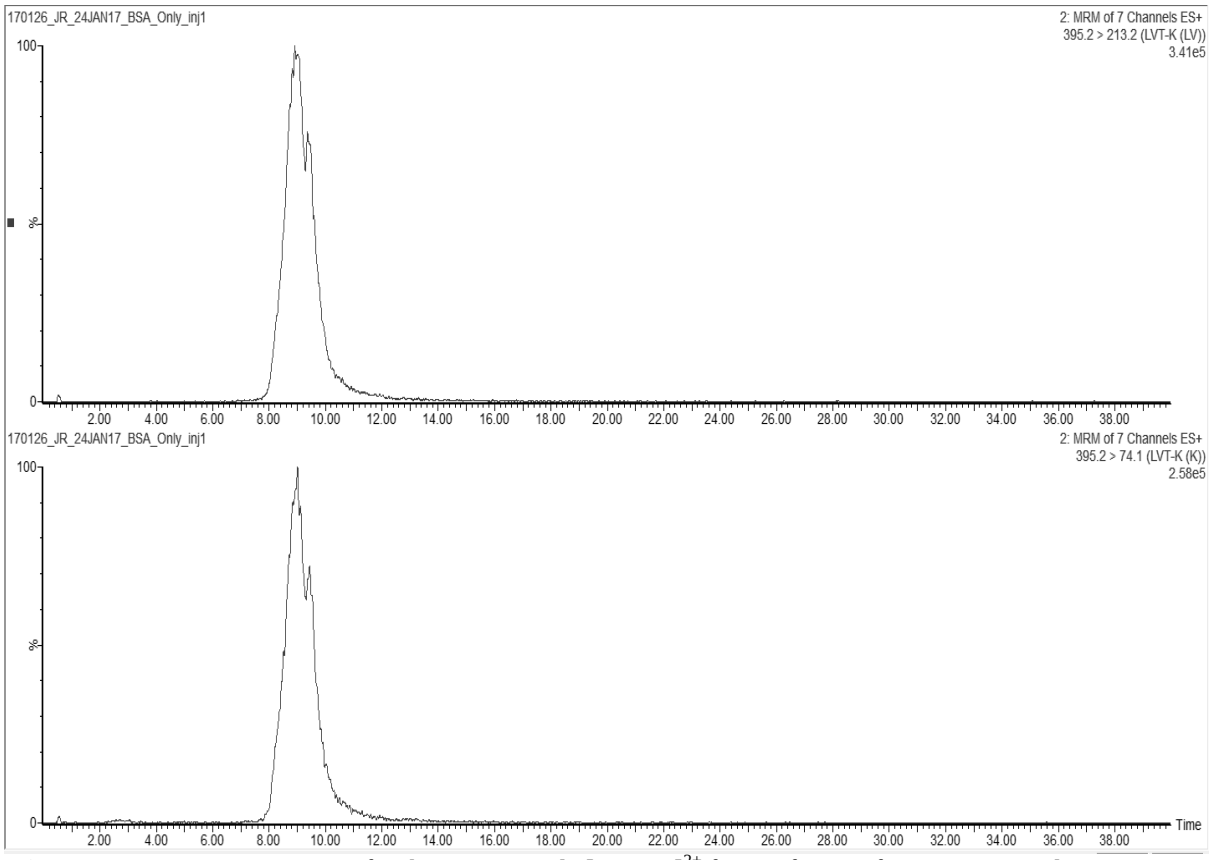


Figure 6. Two MRM transitions for the BSA peptide $[LVT\text{-}K]^{2+}$ from infusion of 5 µM tuning solution

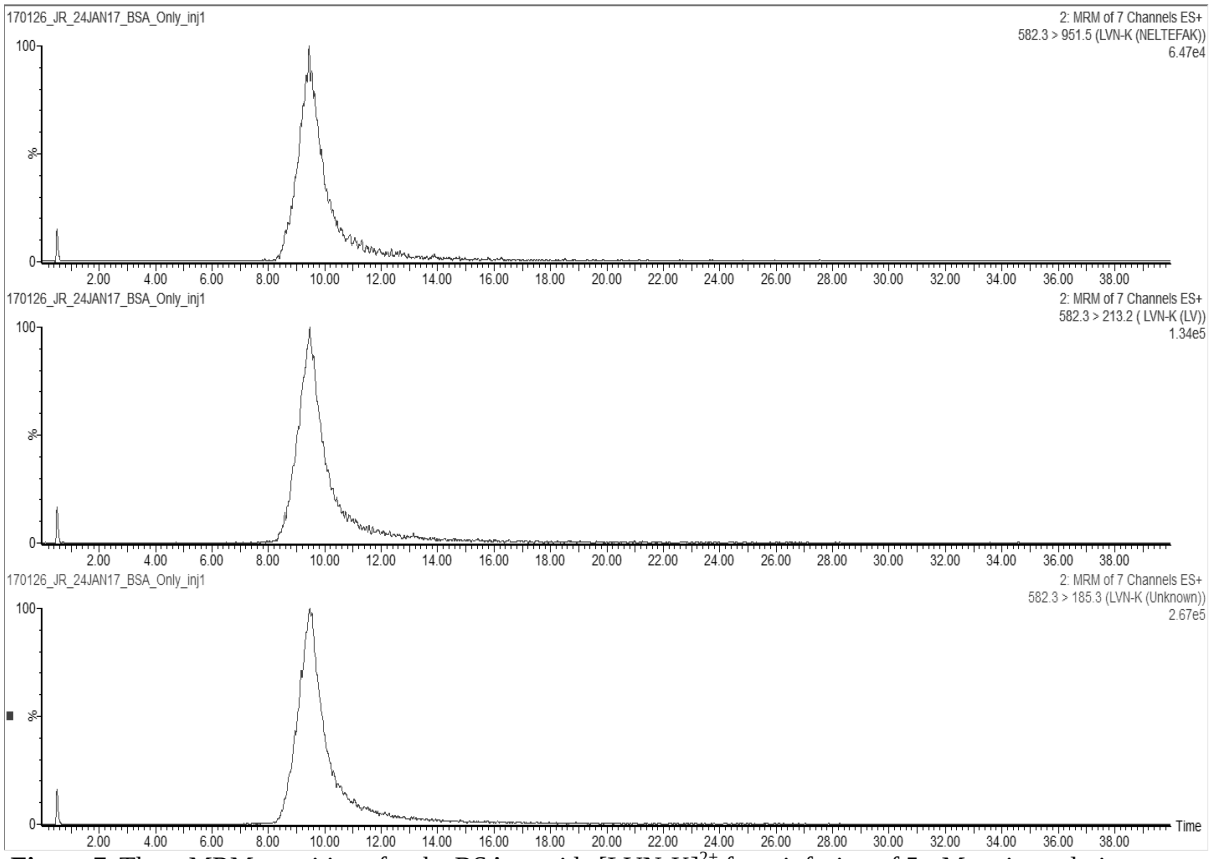


Figure 7. Three MRM transitions for the BSA peptide $[LVN\text{-}K]^{2+}$ from infusion of 5 µM tuning solution

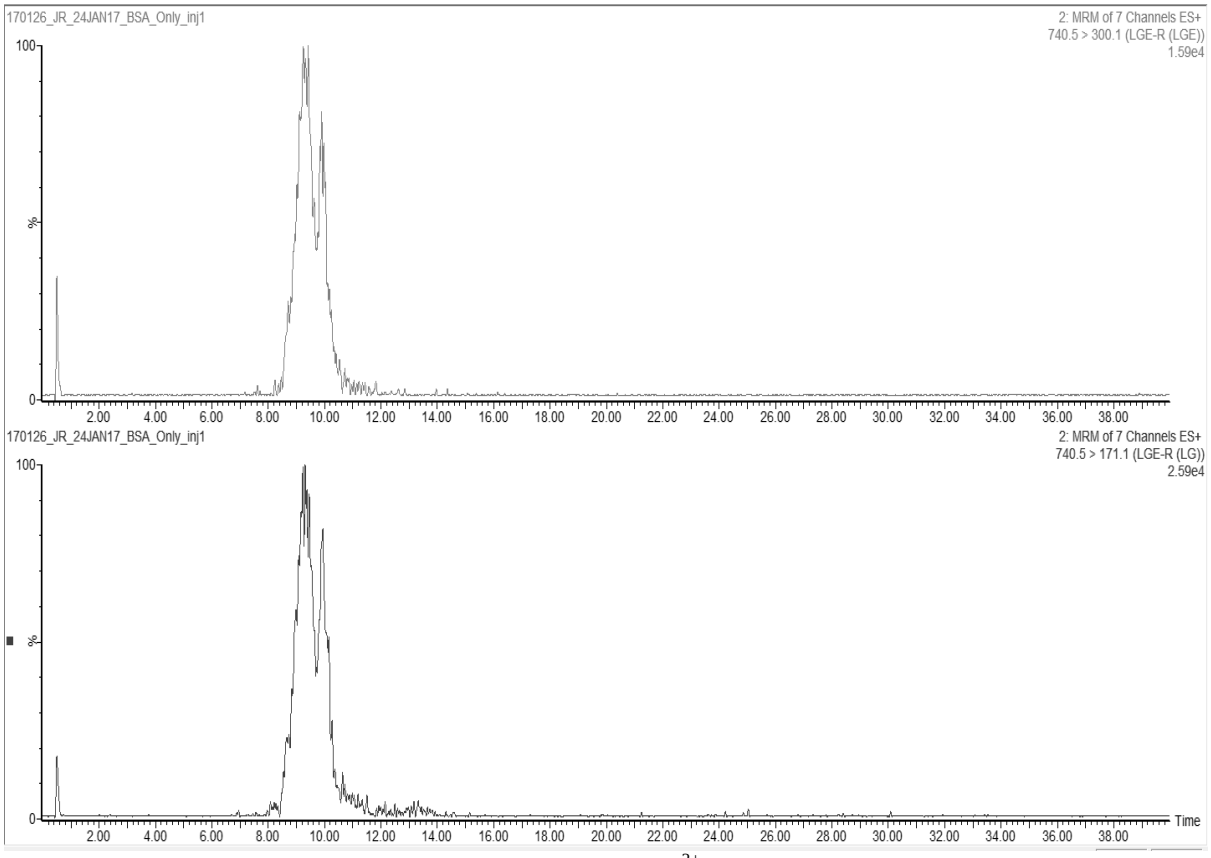


Figure 8. Two MRM transitions for the BSA peptide $[LGE\text{-}R]^{2+}$ from infusion of 5 µM tuning solution

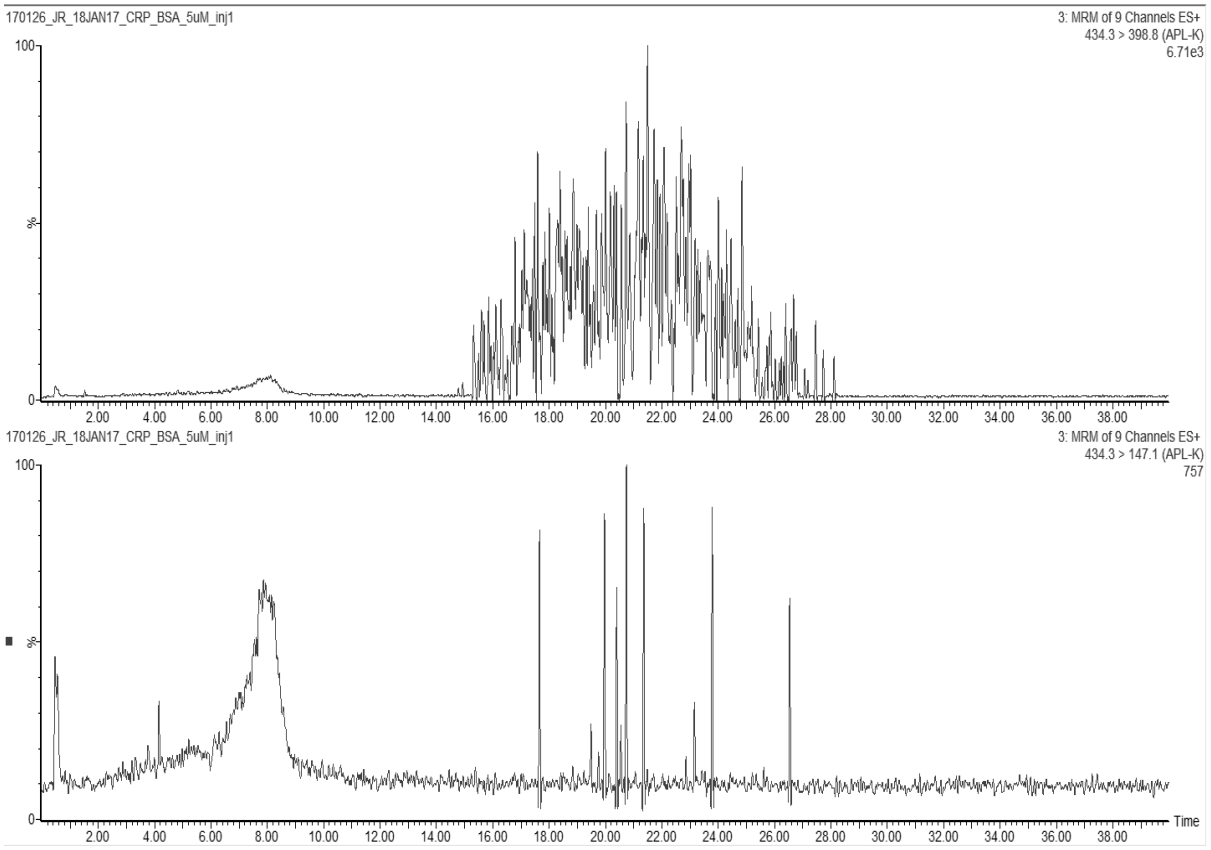


Figure 9. Two MRM transitions for the CRP peptide $[APL\text{-}K]^{2+}$ from infusion of 5 µM tuning solution

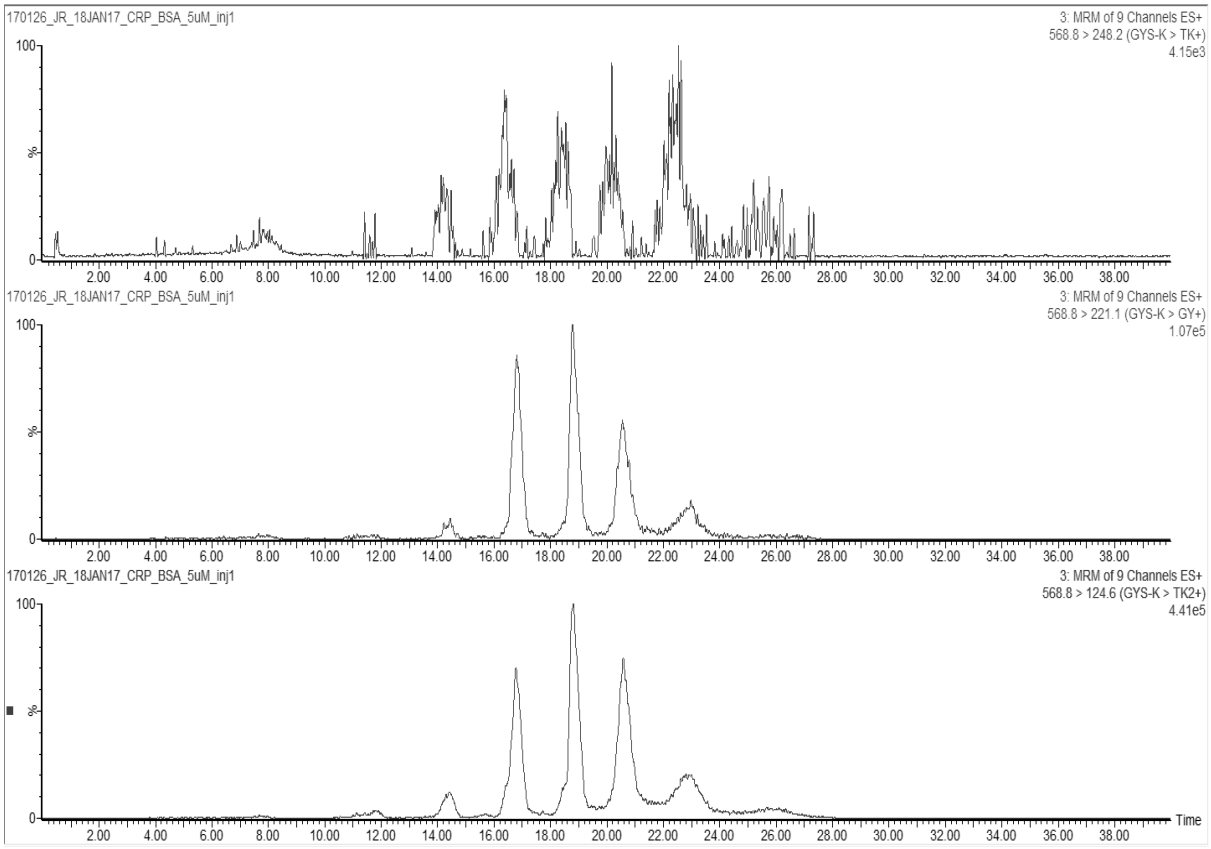


Figure 10. Three MRM transitions for the CRP peptide $[GYS\text{-}K]^{2+}$ from infusion of 5 µM tuning solution

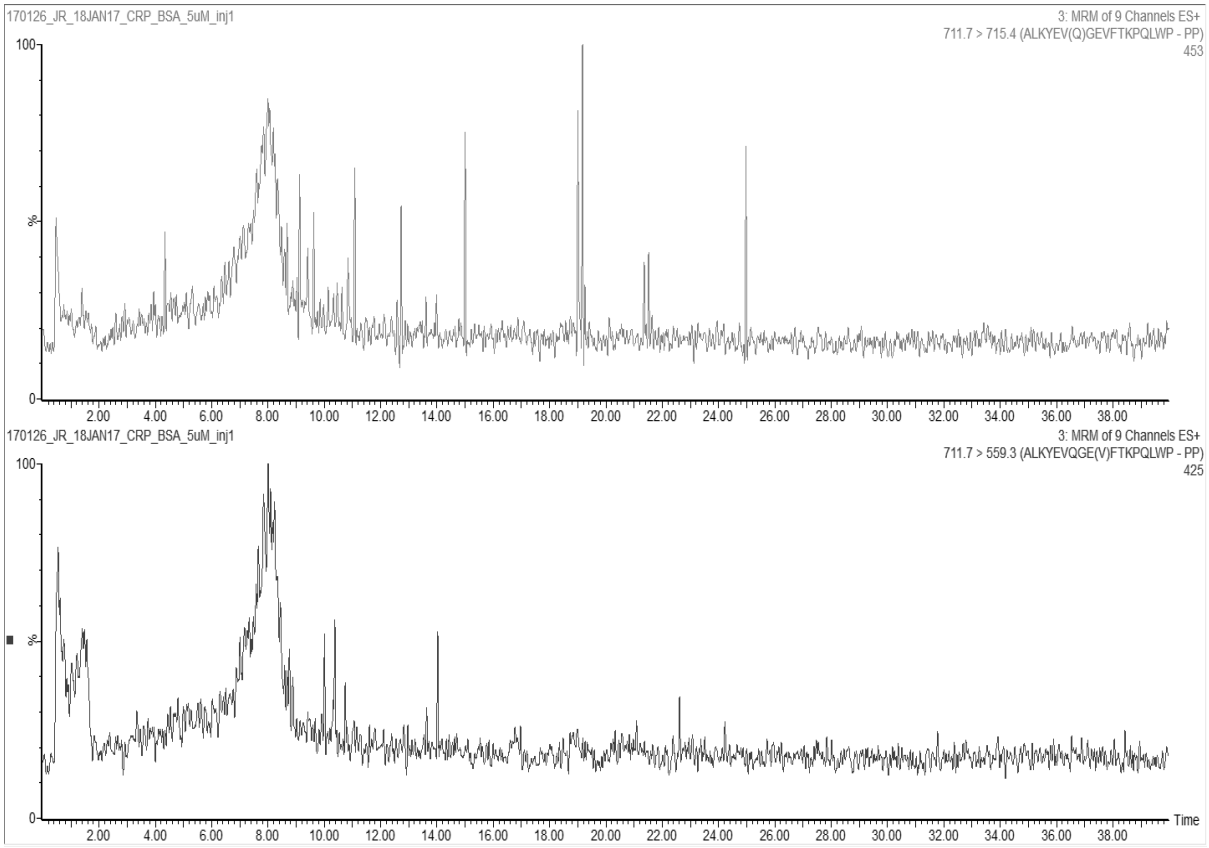


Figure 11. Two MRM transitions for the CRP peptide $[ALK\text{-}P]^{3+}$ from infusion of 5 µM tuning solution

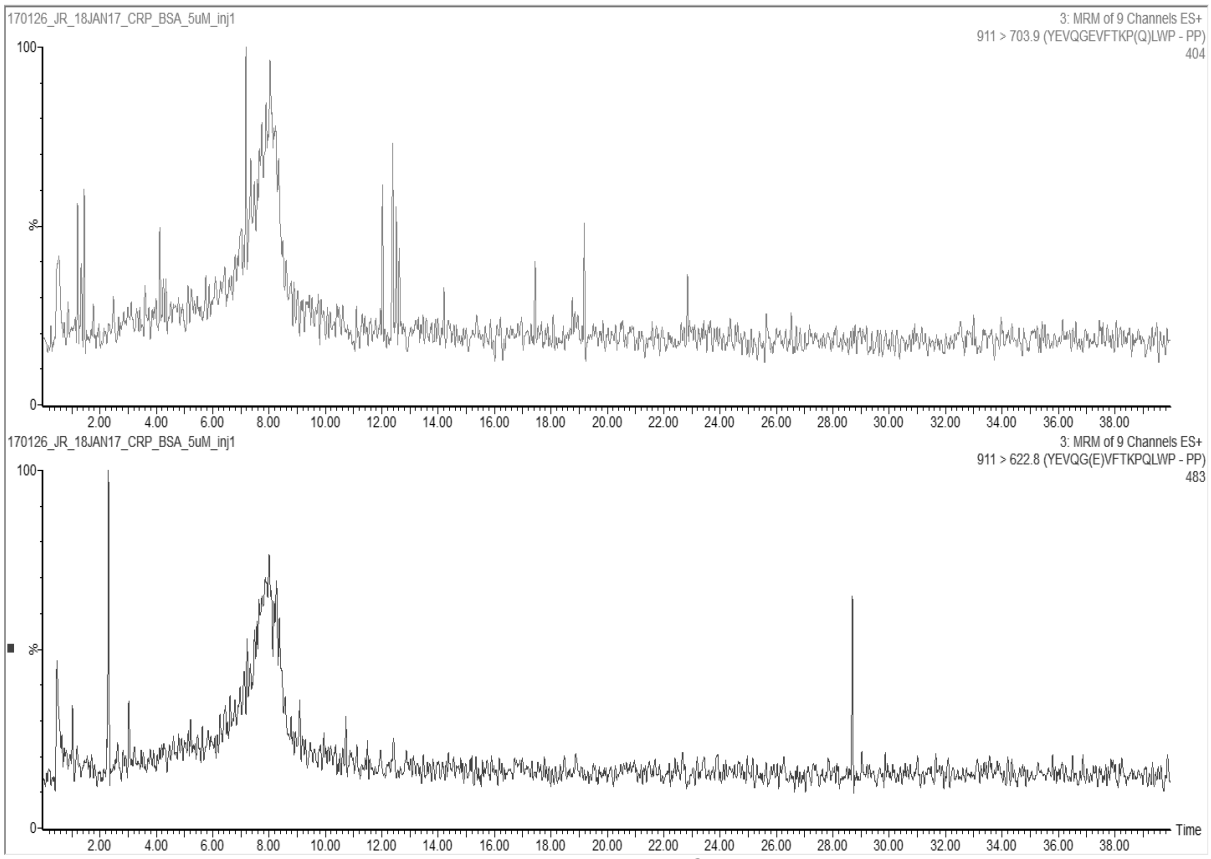


Figure 12. Two MRM transitions for the CRP peptide $[YEV\text{-}P]^{2+}$ from infusion of 5 µM tuning solution

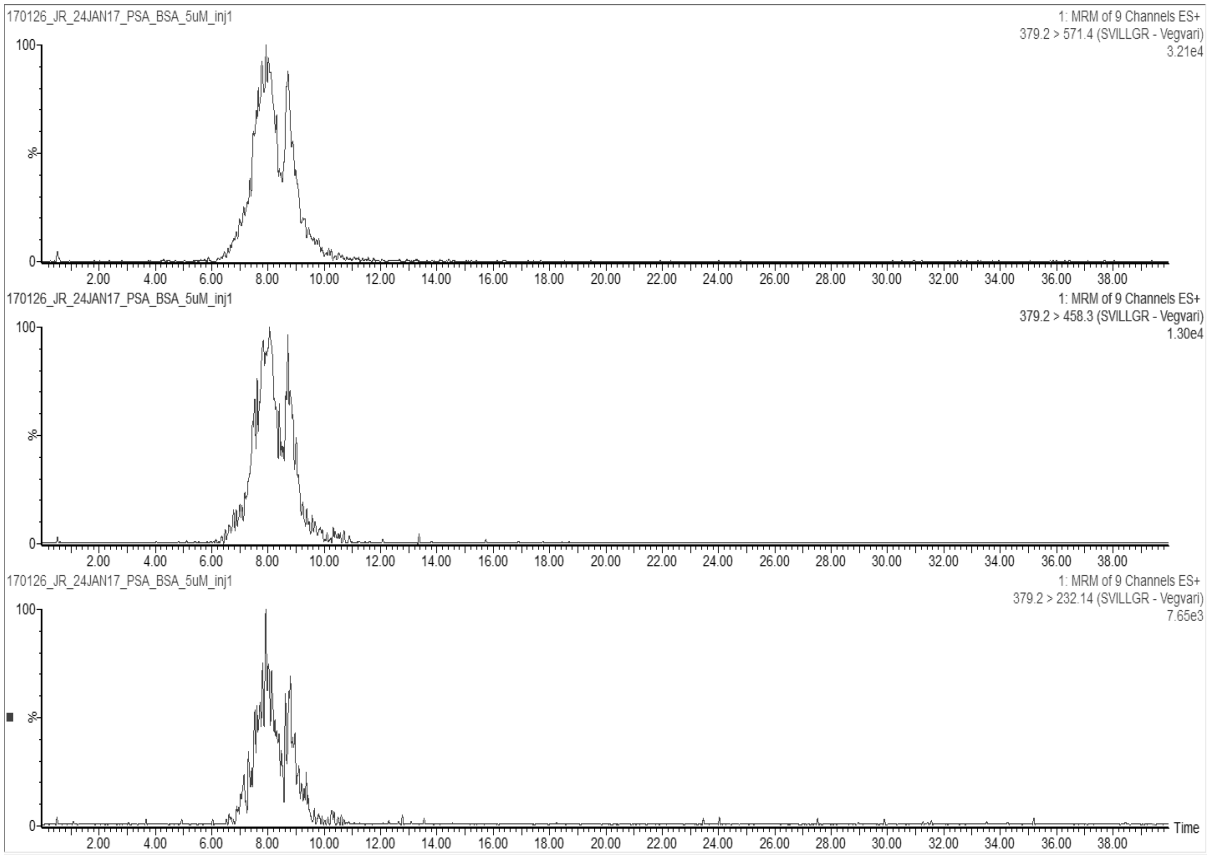


Figure 13. Three MRM transitions for the PSA peptide $[SVI\text{-}R]^{2+}$ from infusion of 5 µM tuning solution

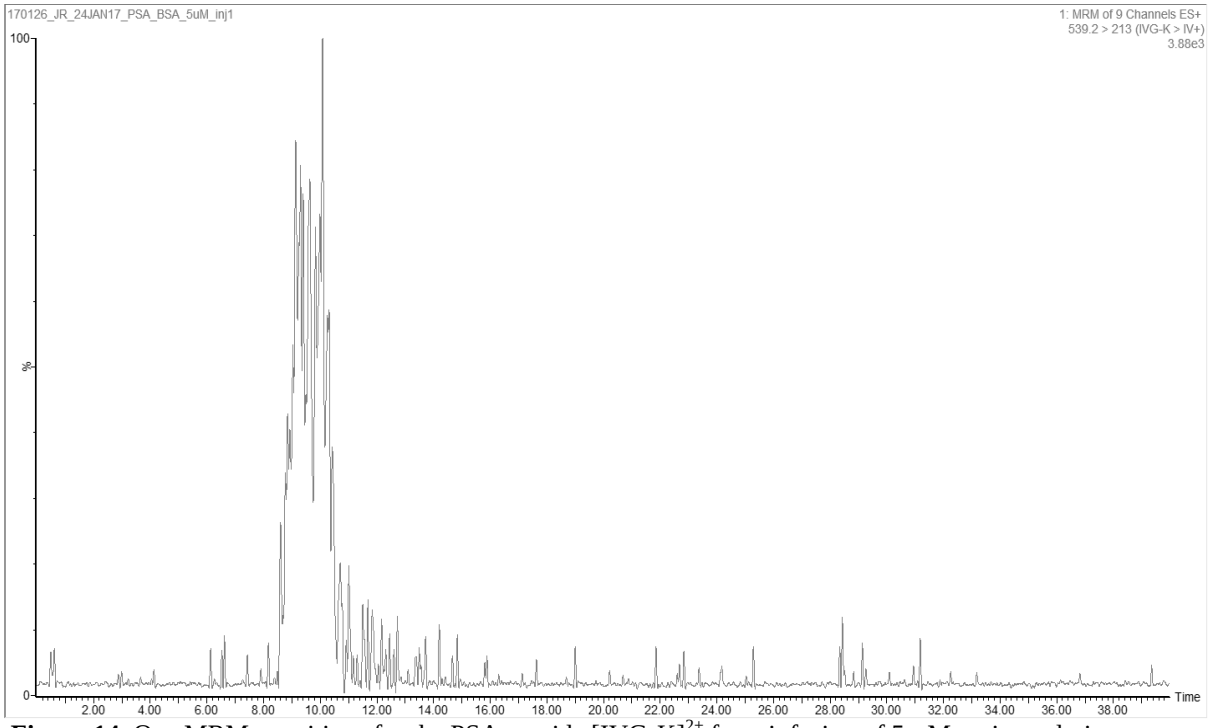


Figure 14. One MRM transitions for the PSA peptide $[IVG\text{-}K]^{2+}$ from infusion of 5 µM tuning solution

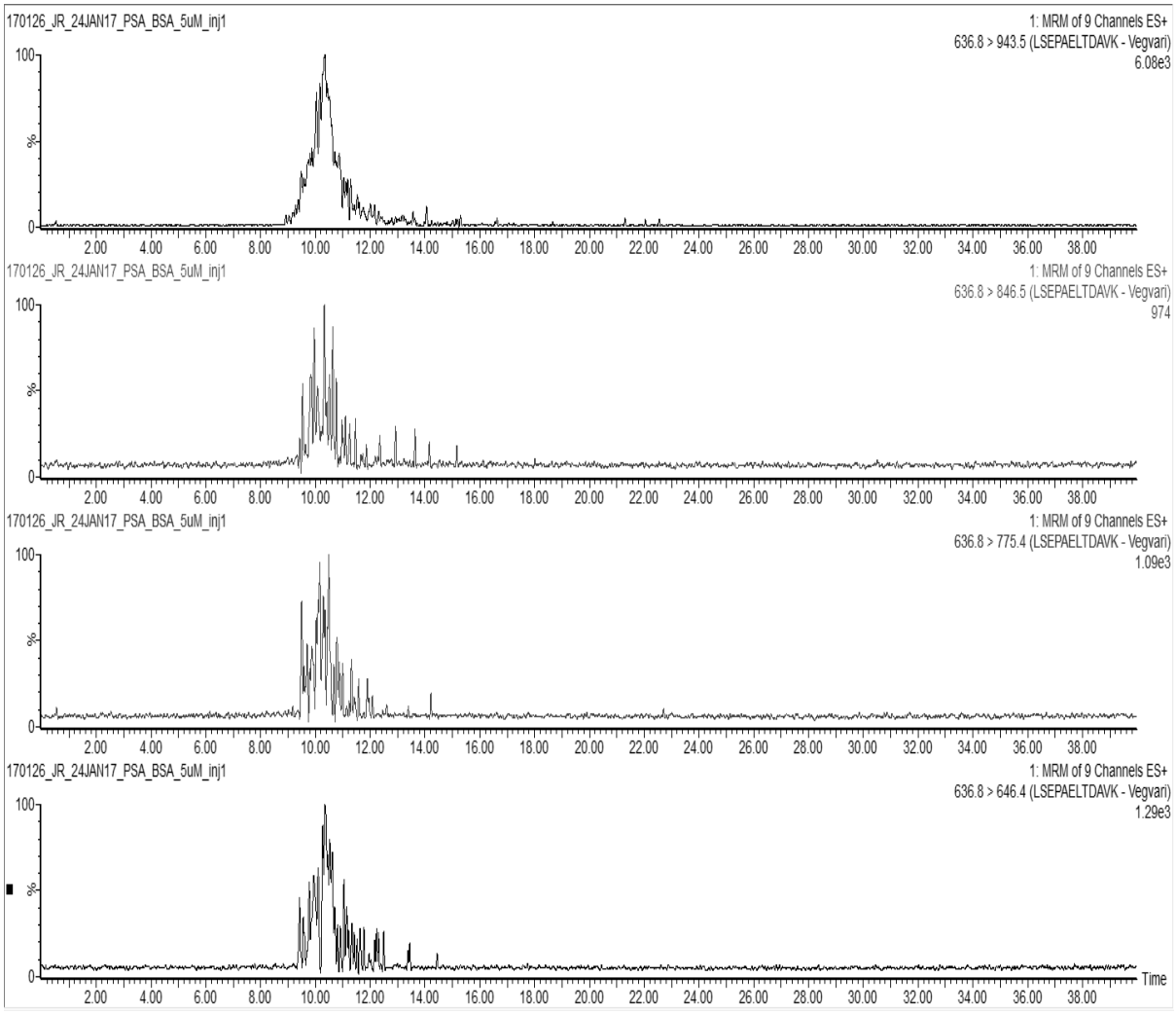


Figure 15. Four MRM transitions for the PSA peptide $[LSE\text{-}K]^{2+}$ from infusion of 5 µM tuning solution

3.4 Preliminary method performance

The preliminary method performance was designed to test the instrument's accuracy, precision and linearity for a range of BSA peptides. The results of the calibration curves prepared to test linearity for the three BSA peptides are shown below in table 8 and figure 16. LOD and LOQ were calculated using the slope of the intercept instead of the more common signal to noise method because the concentration range did not include samples with a low enough concentration to reach S/N = 3.3 or 10.

Table 8. Preliminary method performance results for BSA

Peptide	Range (µg/mL)	Linearity	Average accuracy (%) ± SD	Average precision (%) ± SD	iLOD (µg/mL)	iLOQ (µg/mL)
LVT-K	1-500	0.999	101.6 ± 15.7	1.1 ± 0.8	25.4	77.0
LVN-K	5-250	0.998	102.7± 15.5	1.2 ± 0.9	19.8	59.9
LGE-R	18-500	0.999	97.6 ± 6.9	1.7 ± 1.9	4.2	12.8

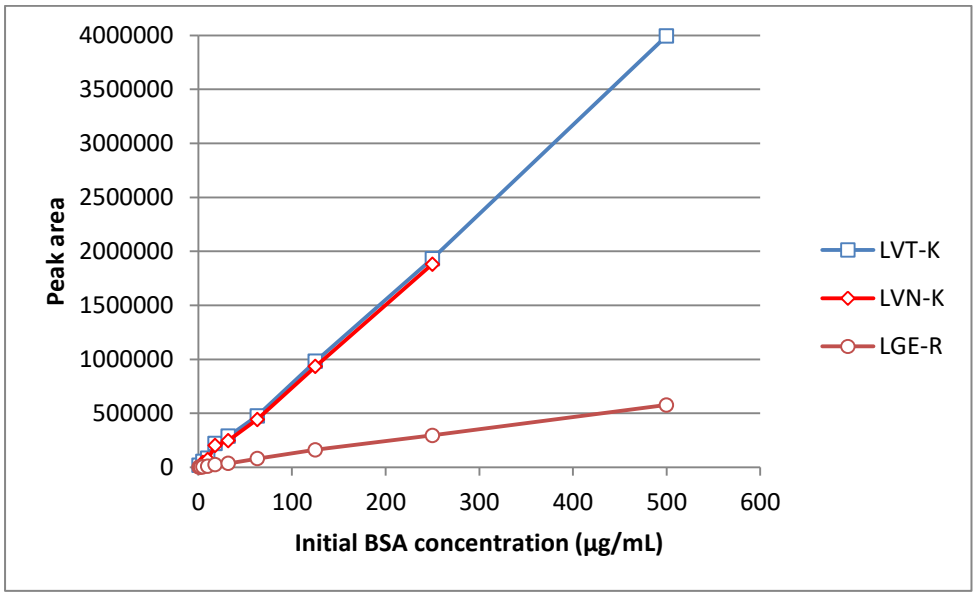


Figure 16. Calibration curves for BSA peptides

All peptides gave excellent linearity, accuracy and precision although this would be expected to as the same sample was being injected twice, and the digest was being carried out at high concentration in buffered solution. However, this was still encouraging and showed that the HILIC-QQQ method was acting reliably over short time periods, and that BSA peptides did exhibit a linear response. For future

method development this would potentially allow for BSA to be used as both a digest control, or as an internal standard for correcting peptide recovery from wastewater and SPE.

3.5 Analysis of wastewater samples

Wastewater pH was measured using five samples, prepared in section 2.6, by measuring the initial pH and pH after the addition of all reagents. The results are shown below in Table 9.

Table 9. Wastewater pH throughout the digestion procedure established in 2.2.2

Step	Sample 1 (pH)	Sample 2 (pH)	Sample 3 (pH)	Sample 4 (pH)	Sample 5 (pH)	Average (pH) ± SD
Initial	7.5	7.5	7.3	7.3	7.3	7.4 ± 0.1
Reduction	8.1	8.0	7.3	7.3	7.4	7.6 ± 0.4
Incubation	7.9	7.9	7.3	7.3	7.3	7.5 ± 0.3
Alkylation	8.0	7.8	7.1	7.1	7.2	7.4 ± 0.4
Incubation	7.9	7.9	6.9	6.8	6.8	7.3 ± 0.5
Enzyme addition	-	-	6.9	6.8	6.8	6.8 ± 0.0
Digest	7.8	7.8	6.9	6.8	6.8	7.2 ± 0.5
Acidification	3.3	3.2	2.6	2.5	2.5	2.8 ± 0.4

The results of the pH stability experiment showed that the pH of wastewater was stable throughout the digestion procedure but was of a lower pH than that of the buffered solution used normally (pH 8.4). However, trypsin is known to be active around pH 7 [40] and because the pH was stable until acidification, where of 0.5% (v/v) of formic acid was still sufficient to reduce the pH to < 4, digest efficiency was not expected to be altered sufficiently to invalidate the procedure.

However, from the first set of digestions it became clear that wastewater would still pose a significant challenge for the digestion method. No BSA or PSA was detected in either of the digests containing wastewater, although the presence of BSA, PSA and CRP in the control digest (without wastewater) showed that the reagents and trypsin were working. There were a series of peaks detected for the CRP peptide GYS-K, which matched was observed in buffer samples both at the time and in method development (section 3.2). The retention times of the GYS-K peak pattern did vary, as did the relative abundance of each peak, but this appeared to be due to a shift in retention time due to matrix effects. Additionally, there did not appear to be a clear difference in GYS-K peptide peak intensity between digest samples 2 and 3, where it would be expected that the addition of 500 nM of CRP would make a difference to peak intensity. Due to the relative similarities in peak shapes and retention times and the

presence of BSA and CRP in the buffer control samples it seemed plausible that CRP was detected in the wastewater samples.

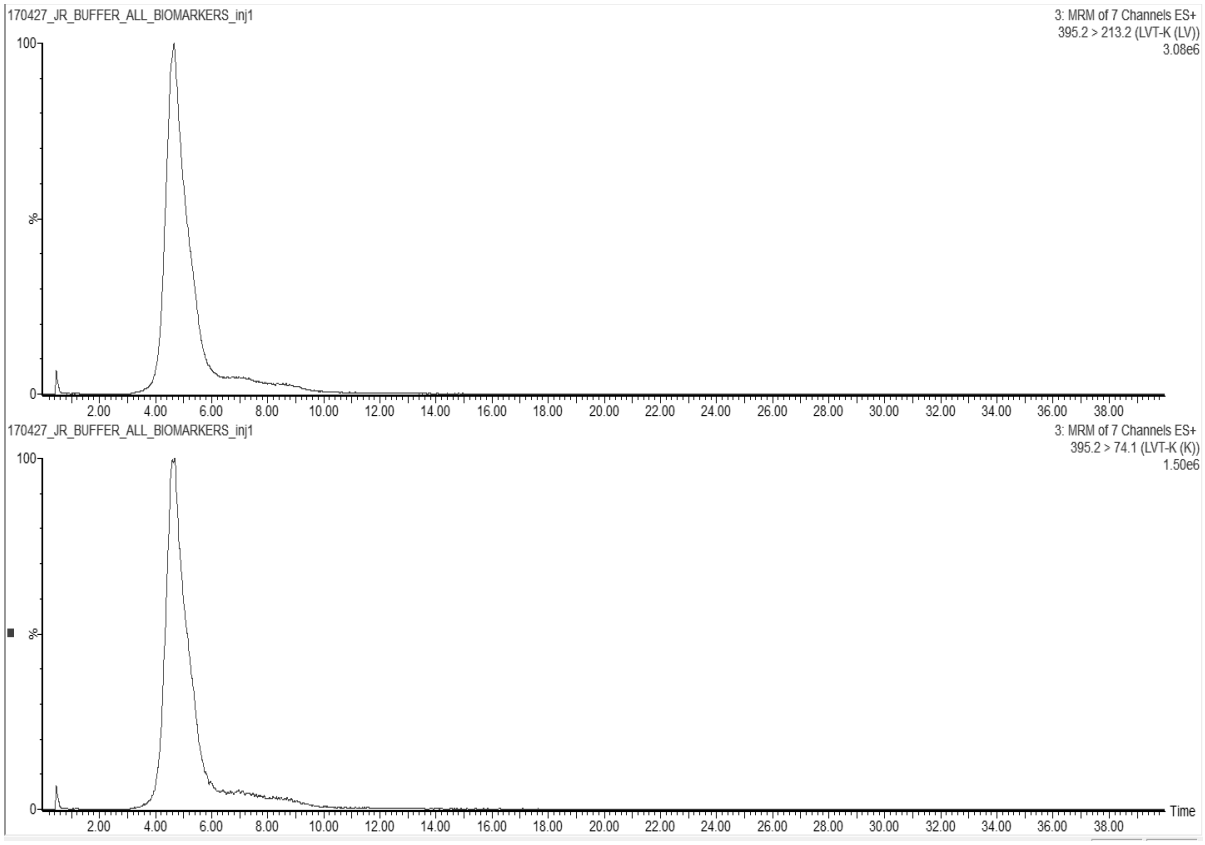


Figure 17. Two MRM transitions for the BSA peptide $[LVT\text{-}K]^{2+}$ in a standard 500 nM buffer digest

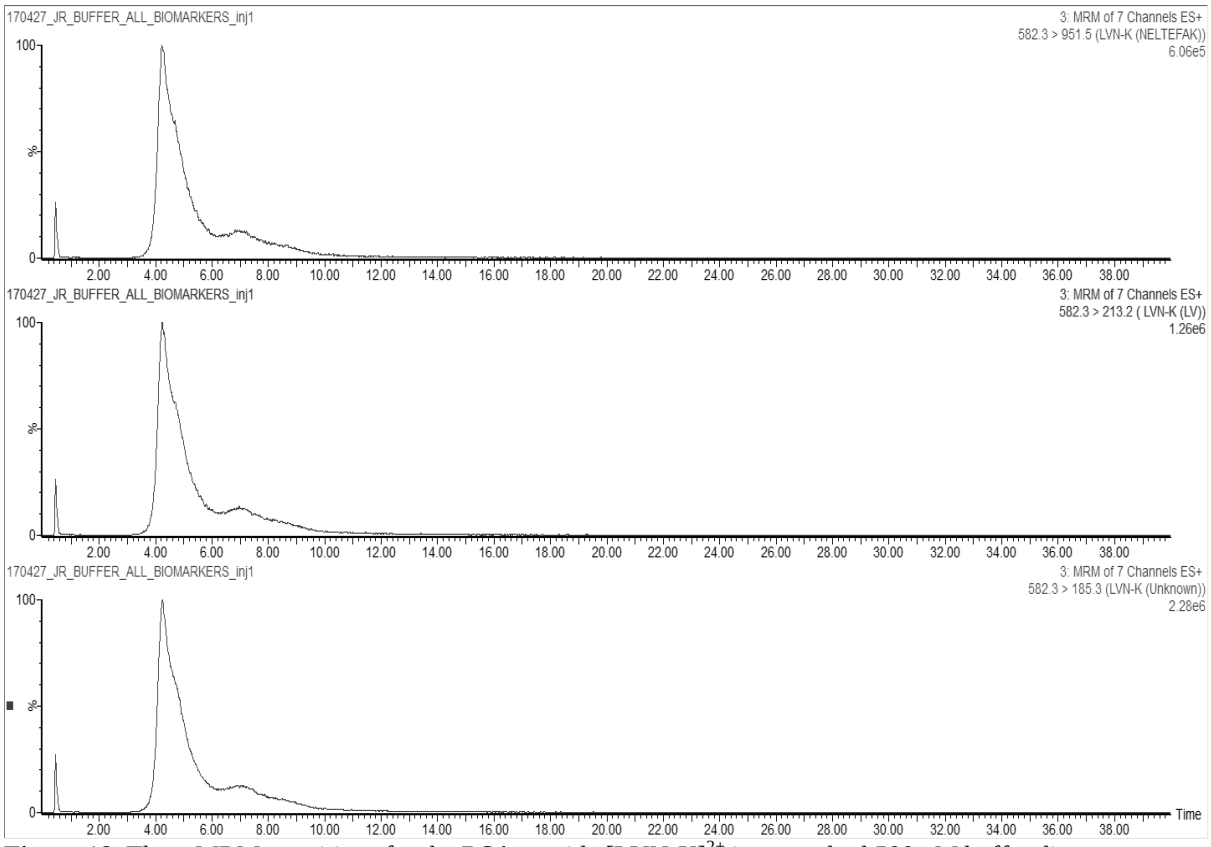


Figure 18. Three MRM transitions for the BSA peptide $[LVN\text{-}K]^{2+}$ in a standard 500 nM buffer digest

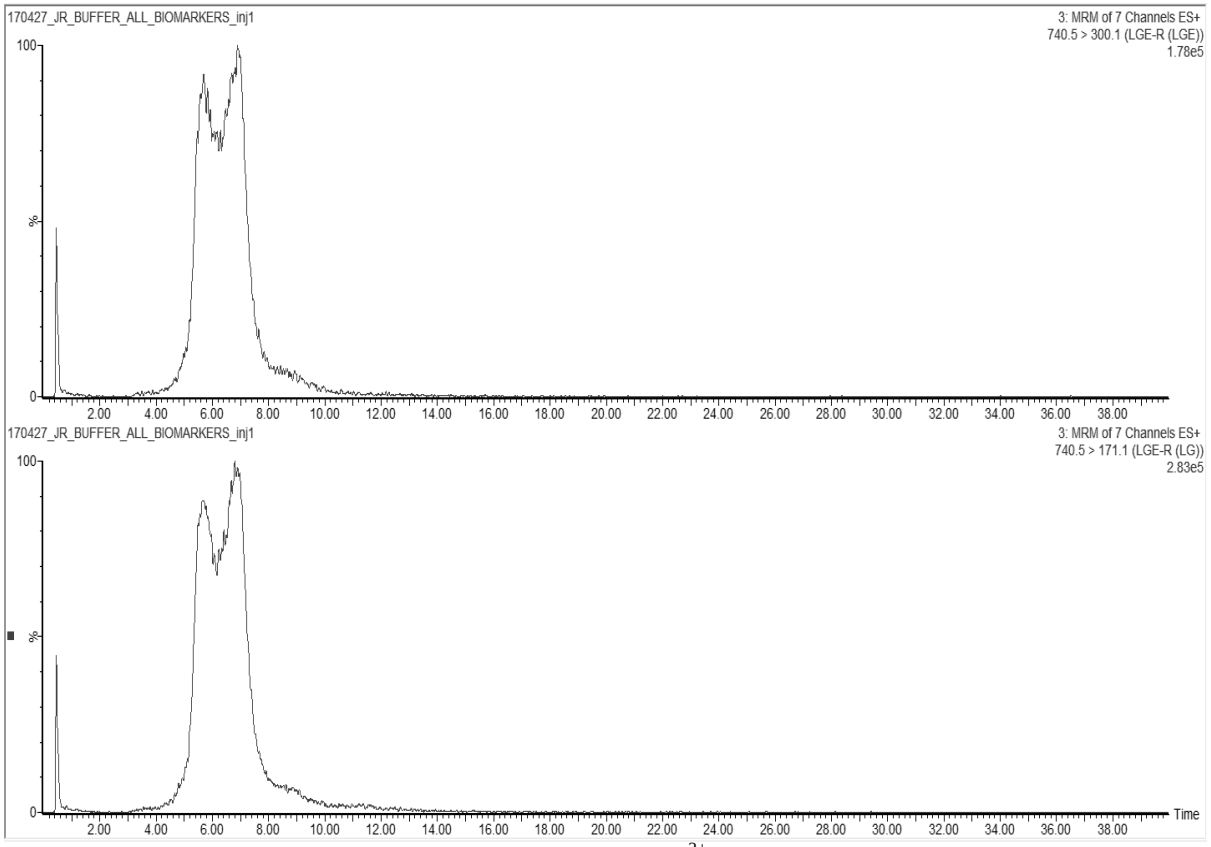


Figure 19. Two MRM transitions for the BSA peptide $[LGE\text{-}R]^{2+}$ in a standard 500 nM buffer digest

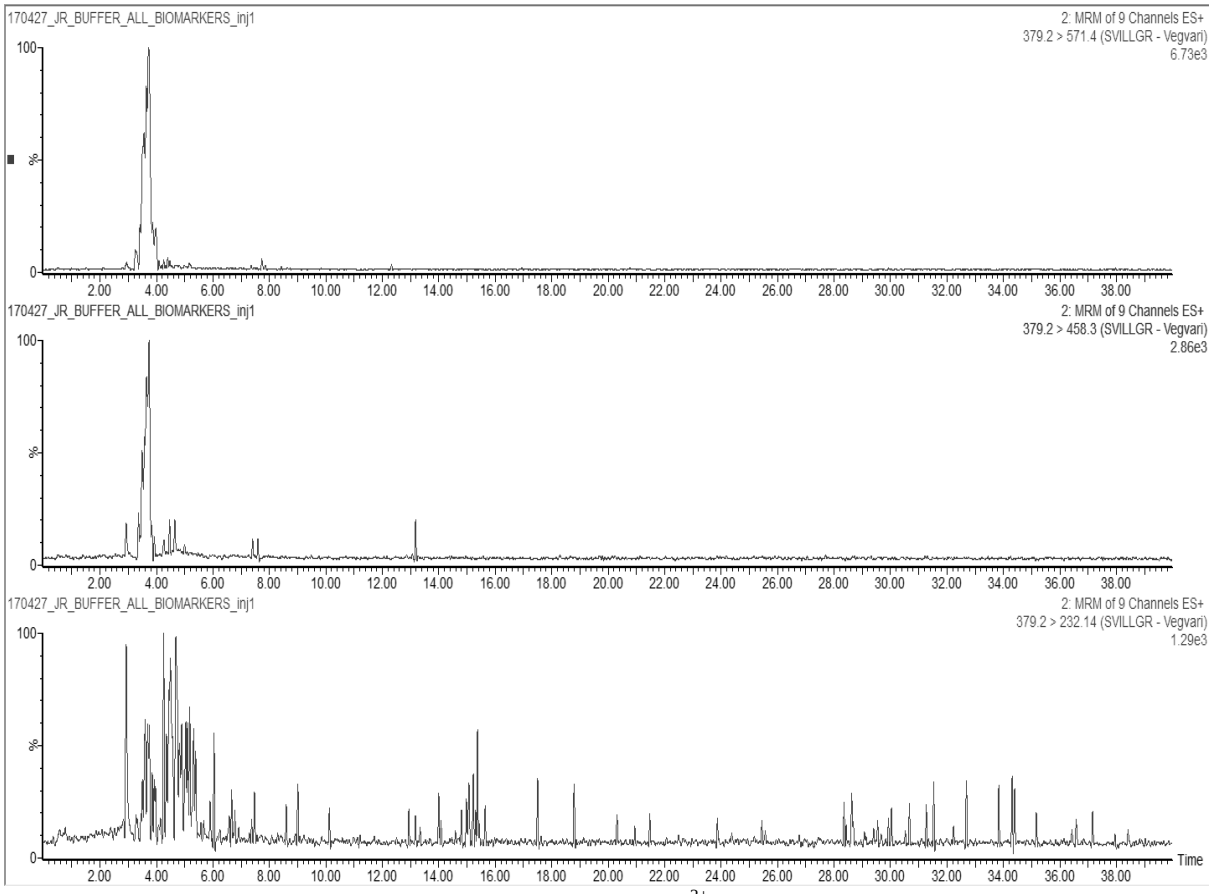


Figure 20. Three MRM transitions for the PSA peptide $[SVI\text{-}R]^{2+}$ in a standard 500 nM buffer digest

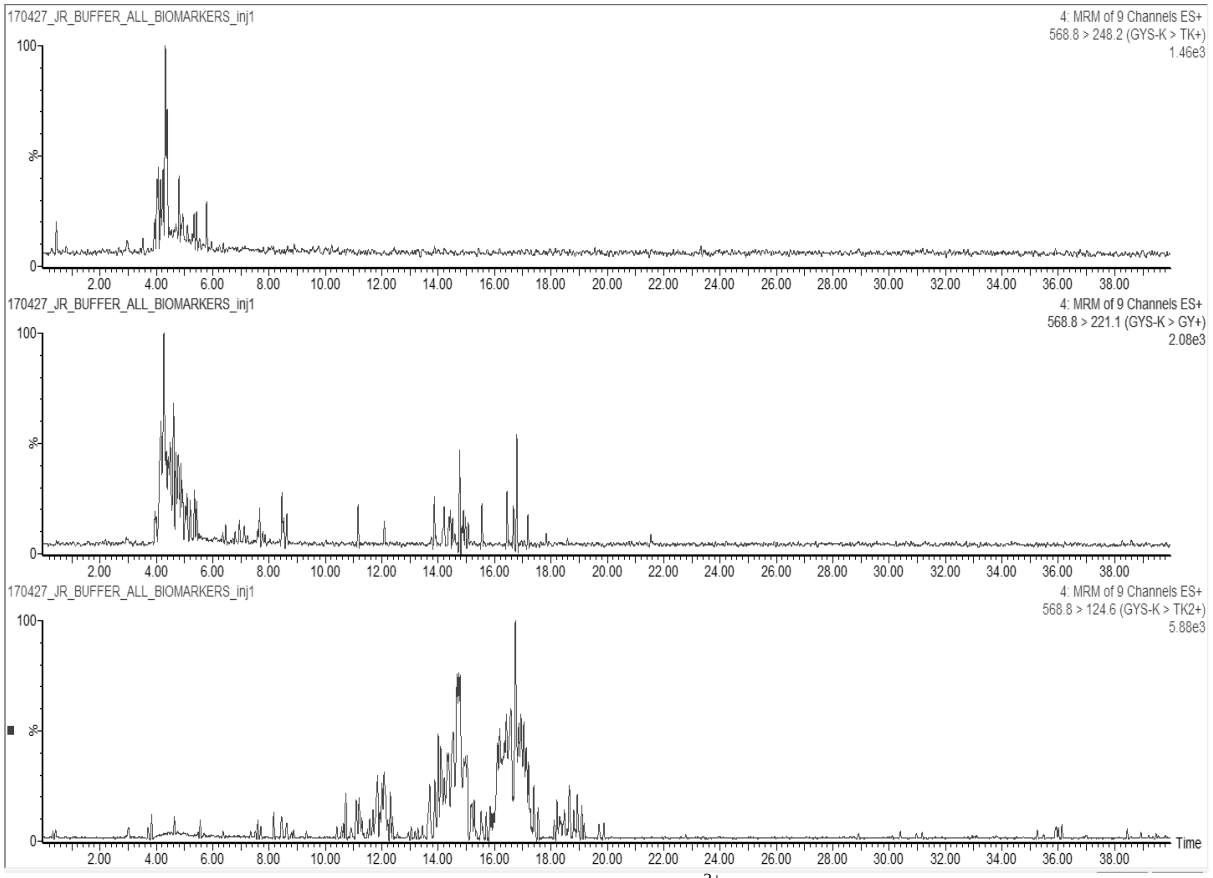


Figure 21. Three MRM transitions for the CRP peptide $[GYS\text{-}K]^{2+}$ in a standard 500 nM buffer digest

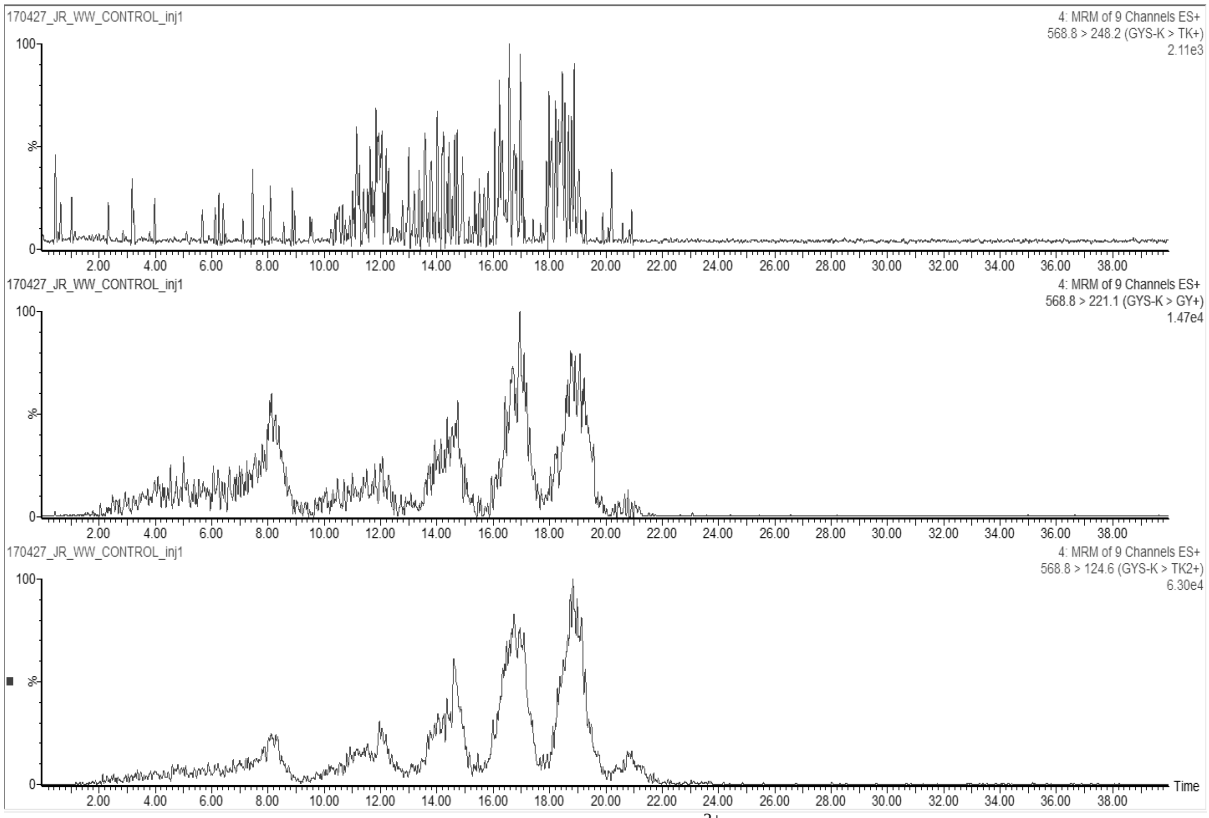


Figure 22. Three MRM transitions for the CRP peptide $[GYS\text{-}K]^{2+}$ in a 100 mL wastewater digest without addition of any proteins

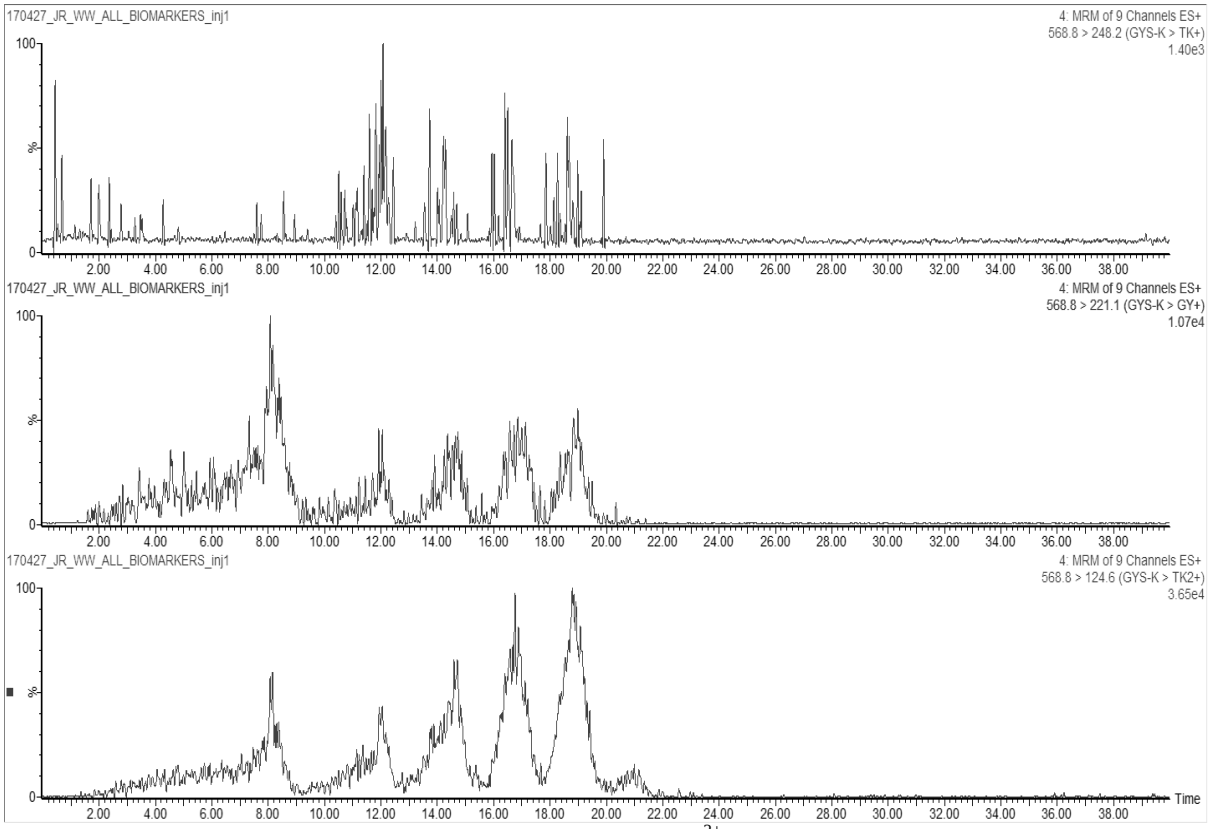


Figure 23. Three MRM transitions for the CRP peptide $[GYS\text{-}K]^{2+}$ in a 100 mL wastewater digest with addition of BSA, CRP and PSA each to a final concentration of 500 nM

The next set of digests prepared used differing concentrations of BSA, CRP and PSA added to samples of wastewater. These digests were prepared to see if BSA or PSA could be detected in wastewater if the concentration at which they were added was increased, as well as looking for repeated detection of the CRP peptide GYS-K in wastewater. The results of these digests are shown in figures 22-27. The increase in BSA concentration from 500 nM to 5 µM was sufficient for it to be detected when added to wastewater (figure 23). This increased the possibility of using BSA as a qualitative measure of whether digestion occurred. PSA remained undetected in wastewater samples despite the increase in concentration. The CRP peptide GYS-K was detected in all wastewater samples, even the wastewater control sample that only added BSA (figure 25), as well as in buffer (figure 24). As with the previous digests there was a shift in retention time for the GYS-K between buffer and wastewater samples, but the same pattern of peaks was observed in both. Once again, the relative abundance of the detected GYS-K peaks varied between buffer and wastewater samples. Likewise, the concentration of CRP added did not appear to change the relative intensity of peak shape of the detected GYS-K peaks. This suggested that either the digest was not working and that the detected peaks were a systematic artefact produced during digestion, or that the amount of CRP is in a large excess relative to the amount of trypsin; meaning that the rate limiting step was the amount of trypsin not the amount of CRP. As BSA is a larger protein with many more disulphide bonds, requiring more extensive reduction and alkylation than CRP, this suggested that there was insufficient trypsin in the digests, rather than insufficient reduction and alkylating reagents.

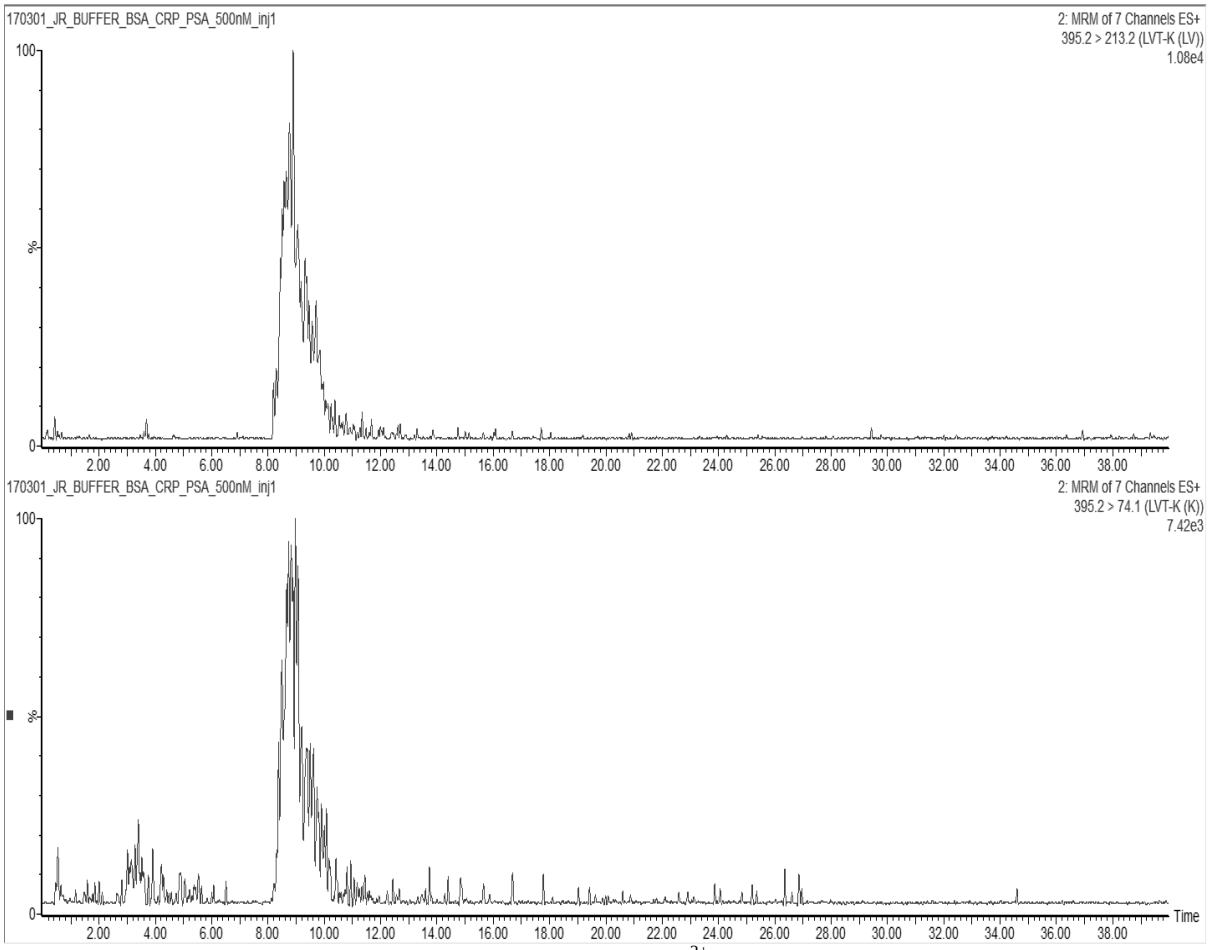


Figure 24. Two MRM transitions for the BSA peptide $[LVT\text{-}K]^{2+}$ in a standard 500 nM buffer digest

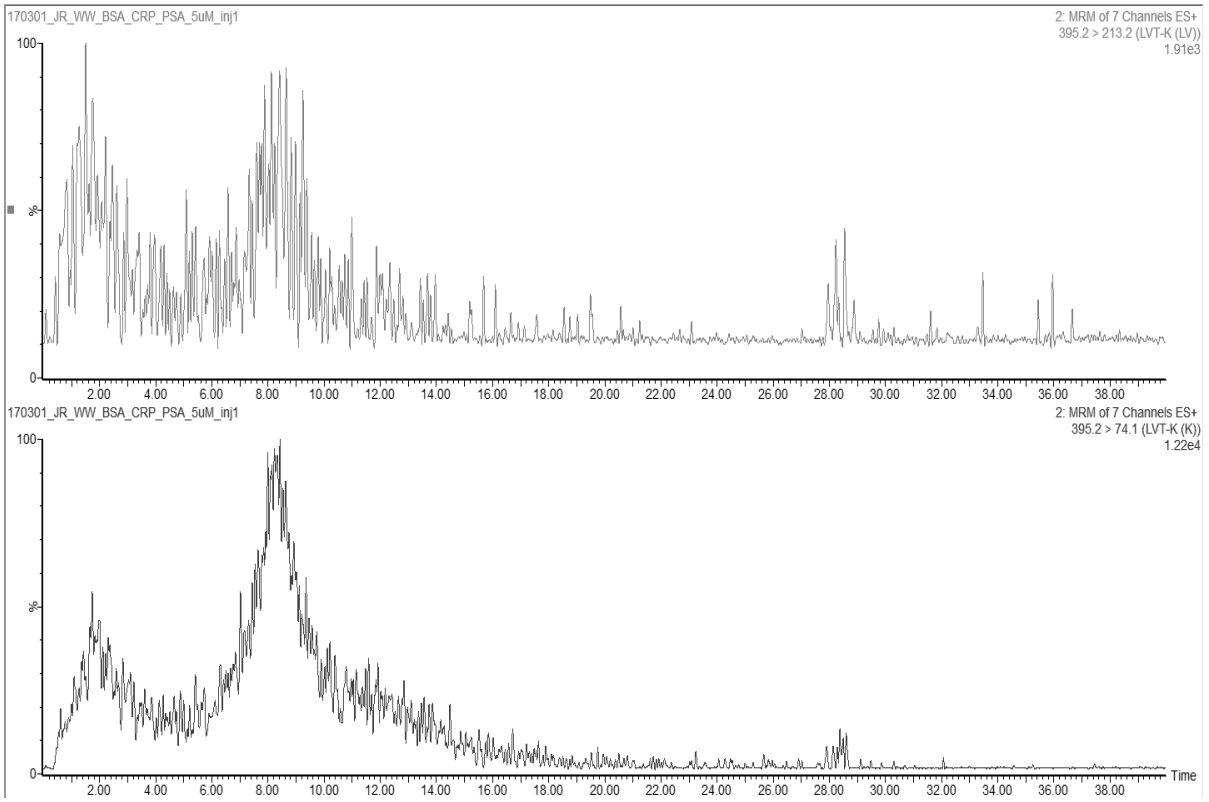


Figure 25. Two MRM transitions for the BSA peptide $[LVT\text{-}K]^{2+}$ in a 100 mL wastewater digest with addition

of BSA, CRP and PSA each to a final concentration of 5 µM

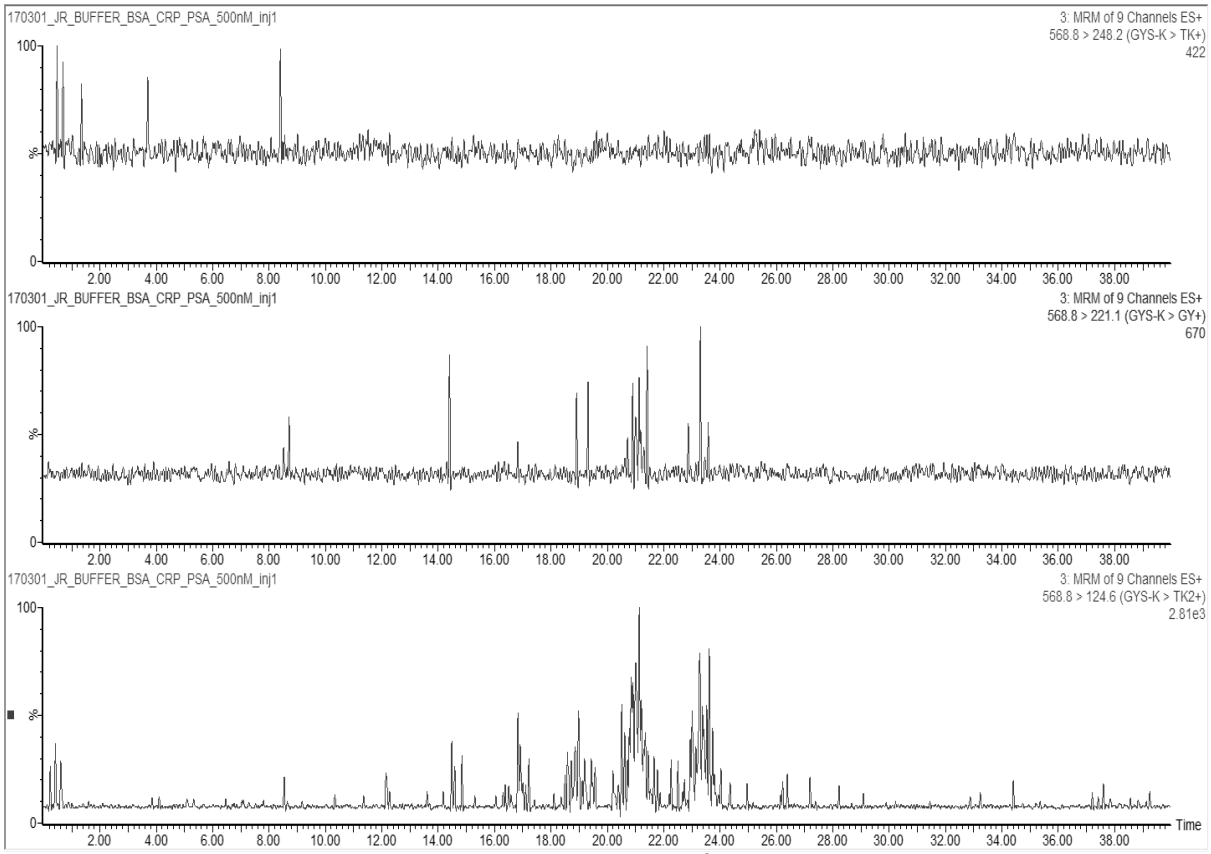


Figure 26. Three MRM transitions for the CRP peptide [GYS-K]$^{2+}$ in a standard 500 nM buffer digest

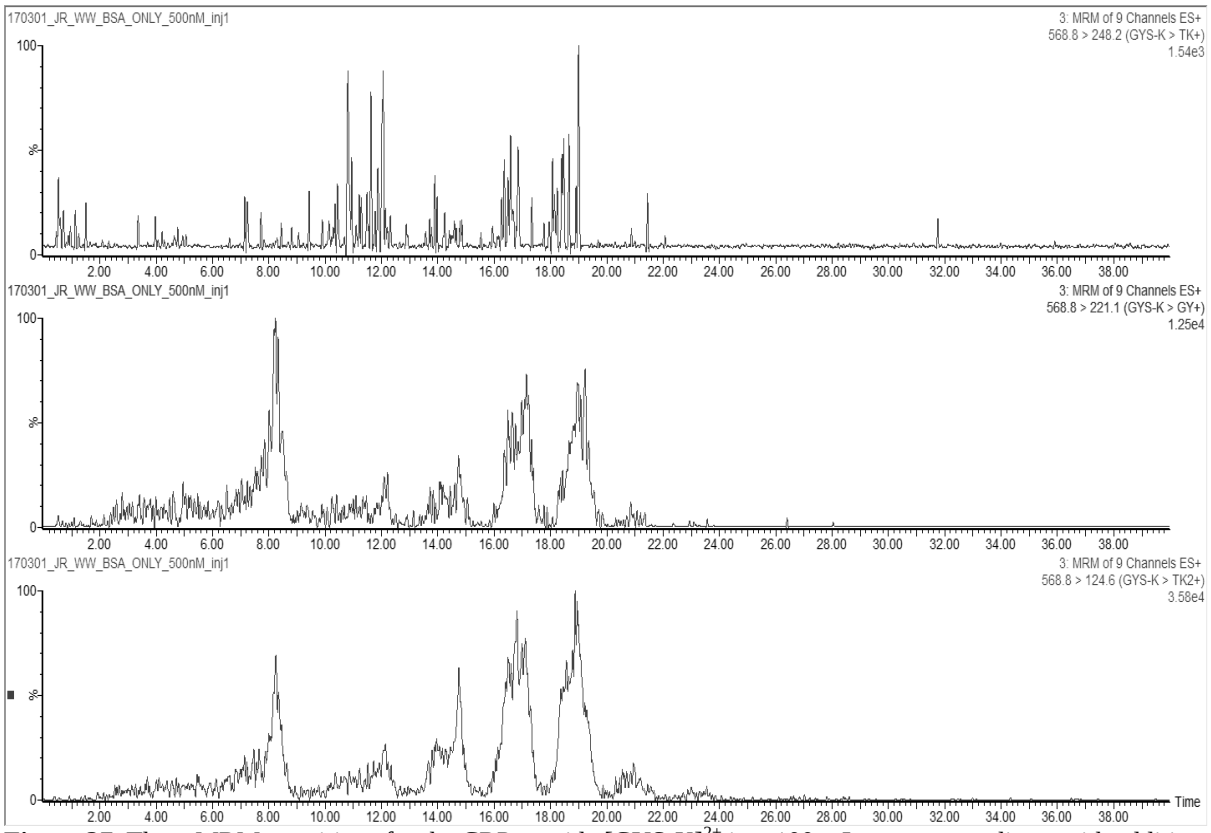


Figure 27. Three MRM transitions for the CRP peptide [GYS-K]$^{2+}$ in a 100 mL wastewater digest with addition of BSA only to a final concentration of 500 nM

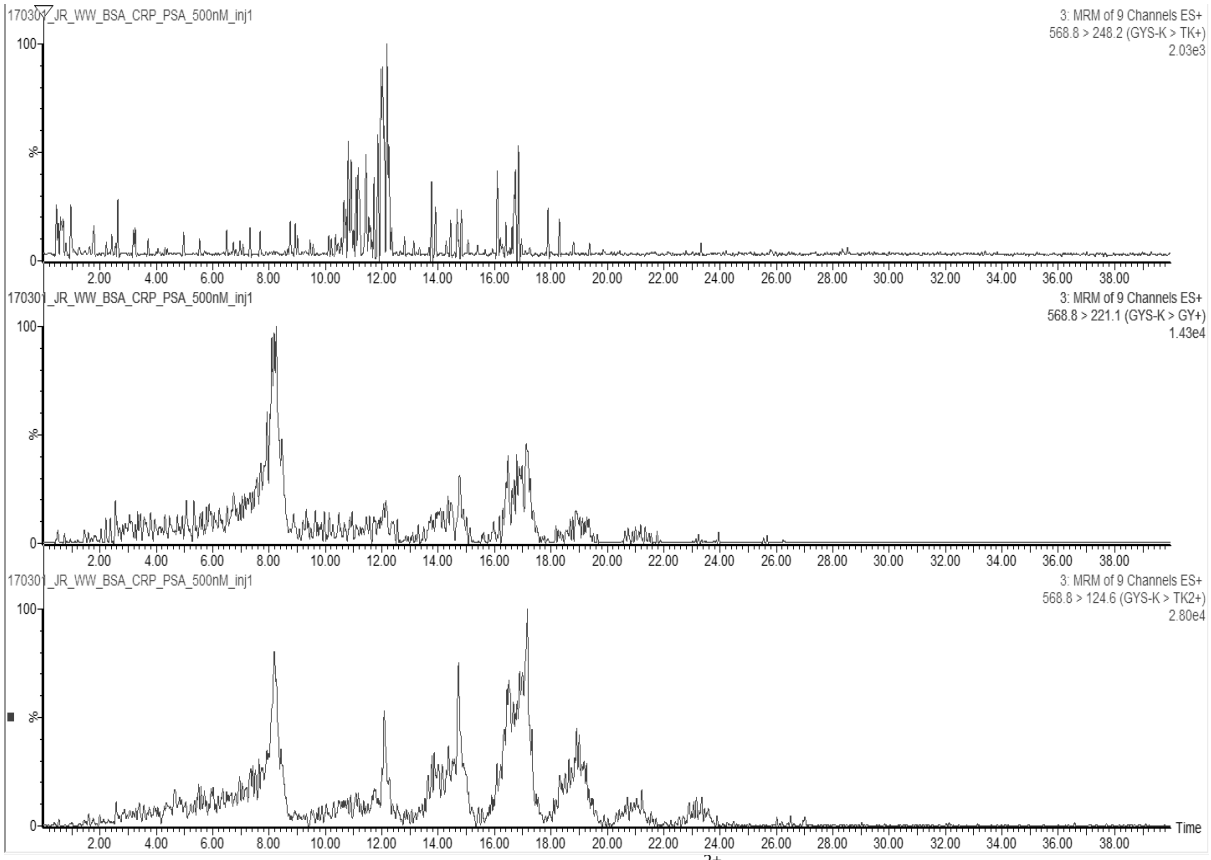


Figure 28. Three MRM transitions for the CRP peptide [GYS-K]$^{2+}$ in a 100 mL wastewater digest with addition of BSA, CRP and PSA each to a final concentration of 500 nM

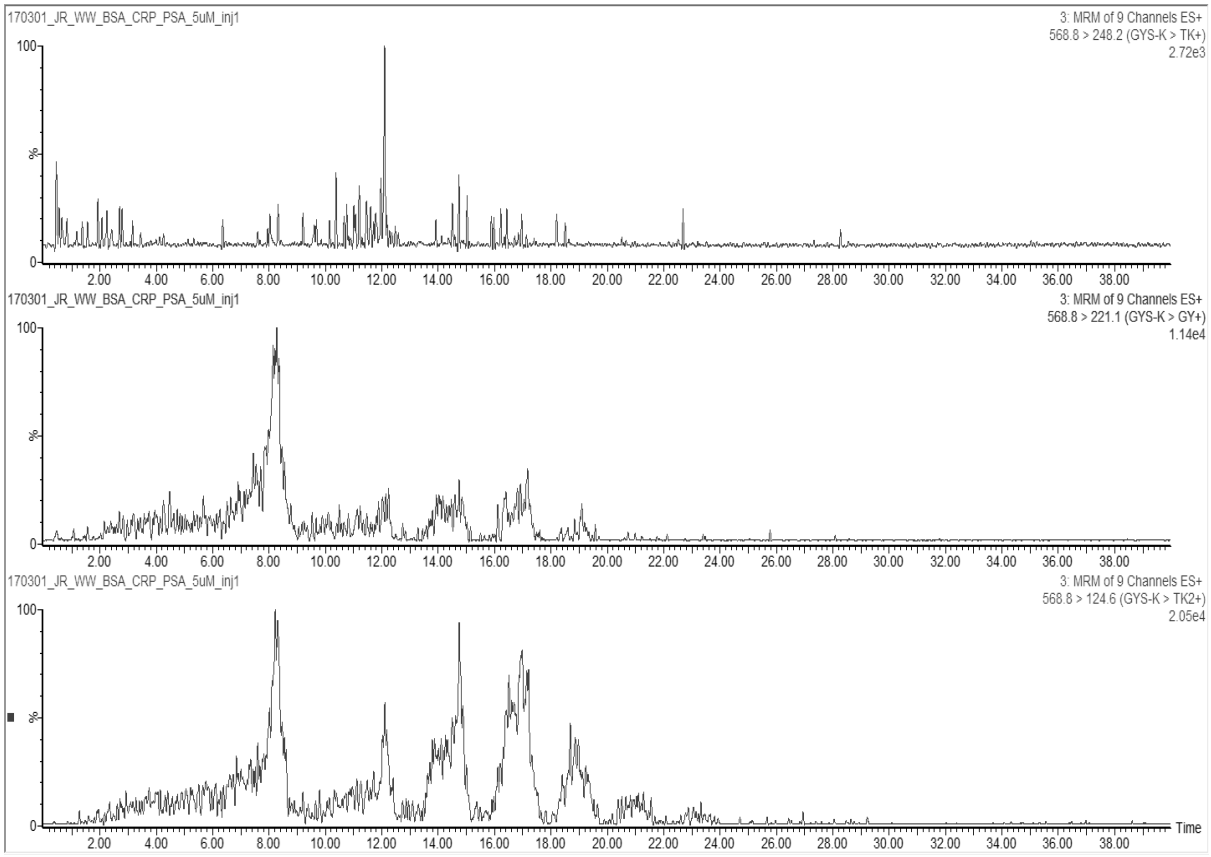


Figure 29. Three MRM transitions for the CRP peptide [GYS-K]$^{2+}$ in a 100 mL wastewater digest with addition of BSA, CRP and PSA each to a final concentration of 5 µM

From the previous digests the amount of trypsin was suspected to be insufficient to effectively digest all the proteins present in wastewater, so the next three digests sought to compare the current methodology with two alternative methodologies. The most obvious was to increase the amount of trypsin added to digests from 25 µL to 1 mL, which would have led to increase CRP concentrations if there had been insufficient trypsin previously. The second digest increased the amount of wastewater used from 100 mL to 500 mL. The rationale was that if increasing the amount of spiked protein did not lead to an observable increase in the amount of GYS-K detected then perhaps the signal suppression is too great, or the SPE recoveries too low for this amount of peptide to make a difference. The final concentration of CRP was therefore increased by increasing the volume of wastewater used instead of the concentration of protein added. However, if trypsin was the limiting factor for digestion then this increase in wastewater volume would not lead to an increase in the amount of GYS-K detected relative to the control digest. For the 500 mL digest the amount of each reagent added was kept the same as for the 100 mL control digest, performed with 25 µL of 20 ng mL^{-1} trypsin. Each digest was performed in duplicate and the results of these digests are shown in figure 28-30.

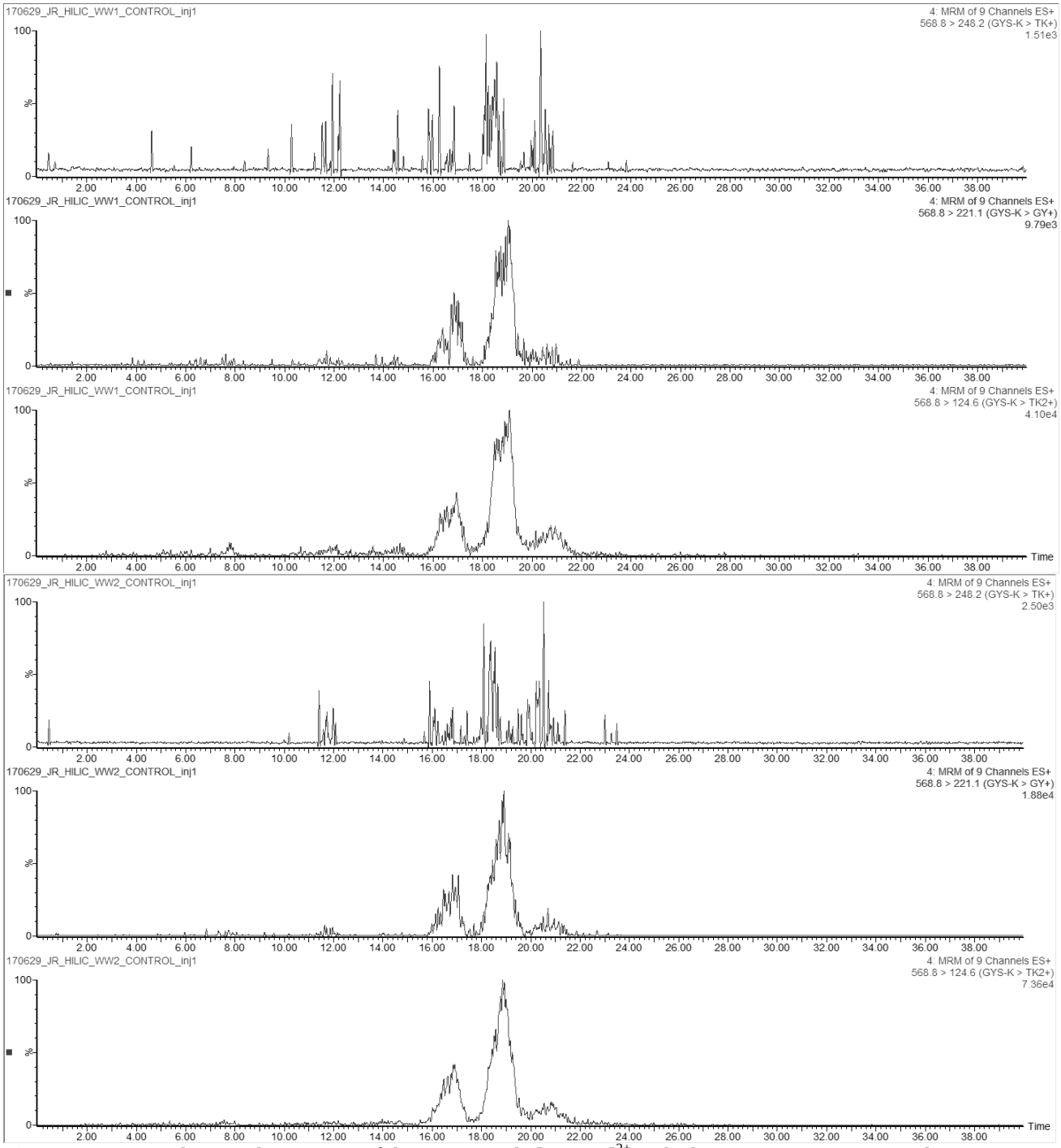


Figure 30. Spectra showing the presence of the CRP peptide $[GYS\text{-}K]^{2+}$ in duplicate 100 mL wastewater digests under standard digest conditions

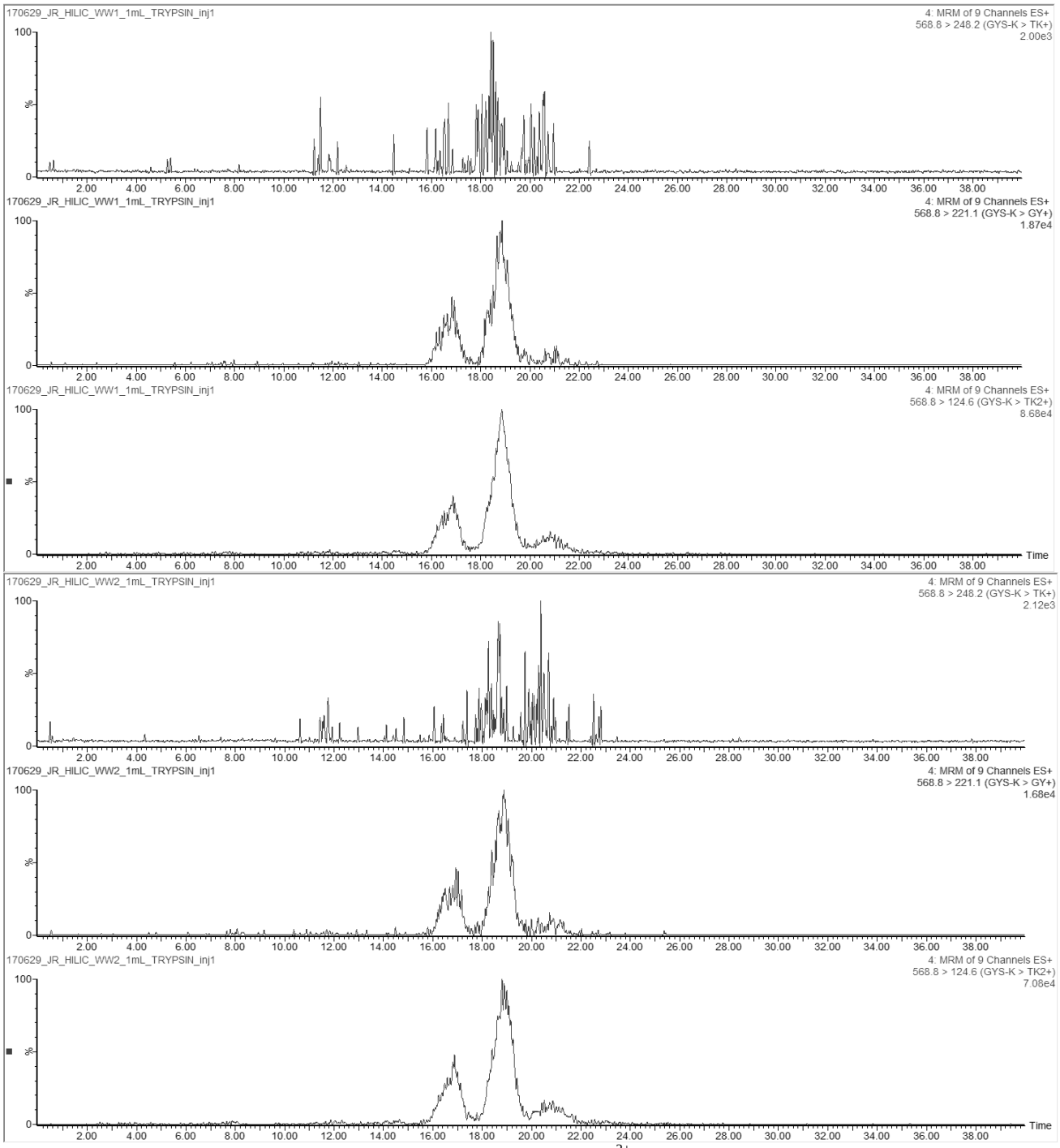


Figure 31. Spectra showing the presence of the CRP peptide $[GYS\text{-}K]^{2+}$ in 100 mL duplicate wastewater digests using 1 mL of 25 µg mL^{-1} trypsin

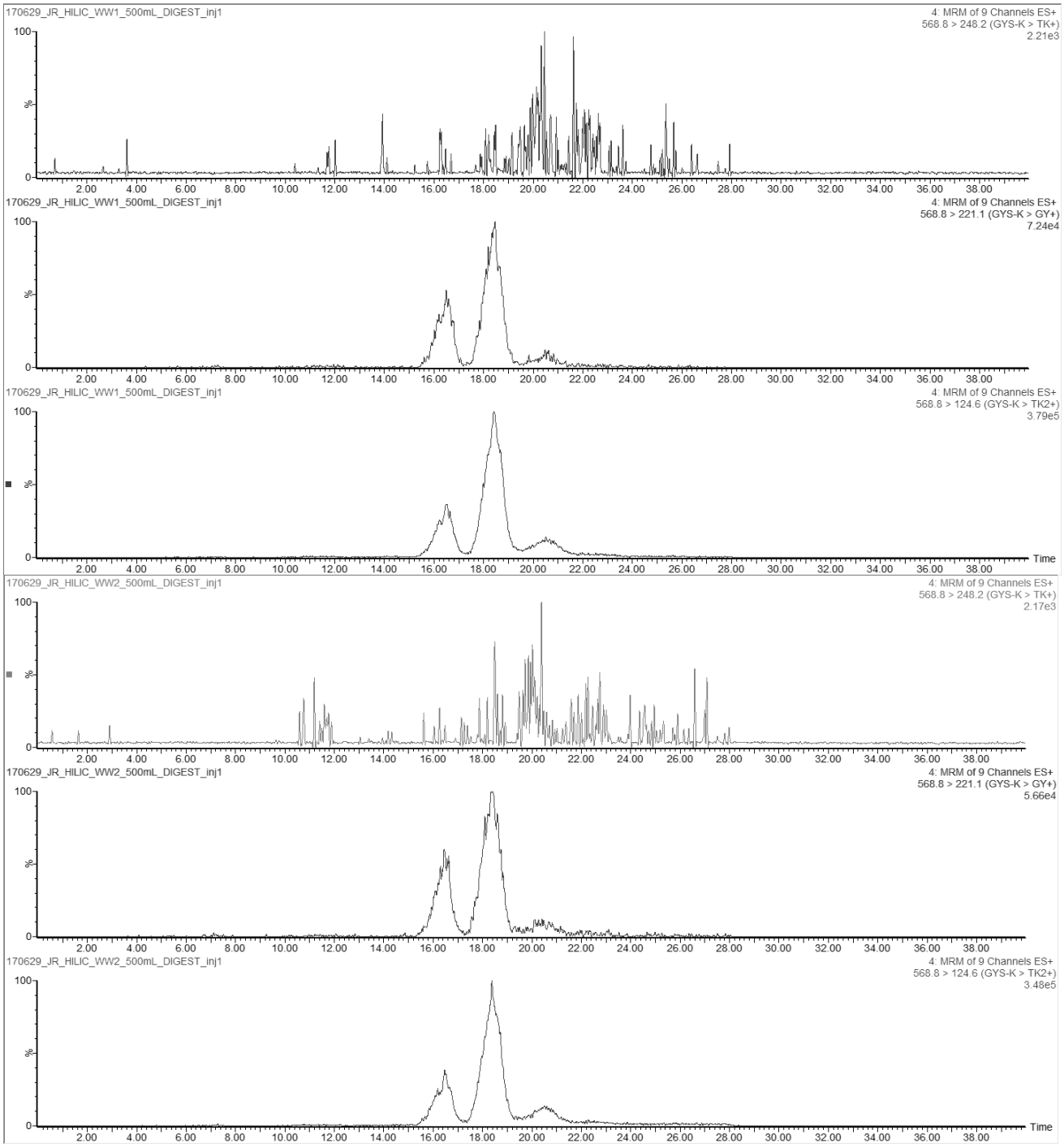


Figure 32. Spectra showing the presence of the CRP peptide GYS-K in 500 mL duplicate wastewater digests, under standard digest conditions

The results of these digests showed that increasing both the amount of wastewater and the amount of trypsin used lead to an increase in the intensity of the GYS-K peaks detected relative to the control. However, this complicated the investigation into how to best detect proteins in wastewater as it suggested that both trypsin and CRP concentration were limiting the amount of GYS-K produced. This can perhaps be explained by the presence of trypsin in wastewater from urinary sources [82], but in humans this is only associated with certain diseases and would not be expected to make up a significant part of the urinary proteome. However, the amount of trypsin used increased 40 times

whereas the amount of wastewater increased only 5 times yet lead to greater peak intensity of GYS-K. Instead, the reason for a lack of sensitivity in response when changing the concentration of CRP added to samples may be due to high signal suppression or poor SPE recoveries. This should be investigated further with a synthetic GYS-K peptide by directly infusing the peptide into the MS post-column during analysis. Alternatively, SPE strategies using charged stationary phases could also be considered and would be expected to give greater selectivity for peptides during extraction, due to the presence of charged lysine or arginine residues after quenching the digest, and reduce matrix effects from wastewater [9].

4. Conclusion

An enzyme digestion method was successfully developed and was able to produce an array of peptides for five different proteins in binary digest of BSA plus one biomarker. The final digestion method made use of commonly available reagents for protein reduction, alkylation and enzyme digestion without the need for either expensive acid-labile surfactants or potentially disruptive reagents like urea. The success of this method allowed for the subsequent development of new methods of analysis, including a digestion method for the analysis of peptides in wastewater and a HILIC-QqQ method for their detection. Analysis of wastewater proved challenging but did result in the repeated detection of CRP in wastewater samples, although PSA and BSA proved elusive even when spiked into samples. The CRP peptide GYS-K was potentially detected in several wastewater digests, although a synthetic peptide reference standard would be needed to confirm this. The detection of a protein of human health in wastewater has not be reported in literature before and would represent an important step forwards for developing new methods to monitor human health. Future innovations should build towards the development and integration of proteomics into the established WBE workflow to enable a deeper understanding of public health.

Acknowledgments

The support of the Leverhulme Trust (Project No RPG-2013-297) and Engineering and Physical Sciences Research Council (Project No EP/P028403/1) is greatly appreciated.

References

[1] Rice, J. and Kasprzyk-Hordern, B., 2019. A new paradigm in public health assessment: Water fingerprinting for protein markers of public health using mass spectrometry. Trends in Analytical Chemistry.
[2] Sims, N., Rice, J. and Kasprzyk-Hordern, B., 2019. An ultra-high-performance liquid chromatography tandem mass spectrometry method for oxidative stress biomarker analysis in wastewater. *Analytical and Bioanalytical Chemistry,* 411(11), pp. 2261-2271.
[3] Baz-Lomba, J.A., Salvatore, S., Gracia-Lor, E., Bade, R., Castiglioni, S., Castrignano, E., Causanilles, A., Hernandez, F., Kasprzyk-Hordern, B., Kinyua, J., McCall, A.K., van Nuijs, A., Ort, C., Plosz, B.G., Ramin, P., Reid, M., Rousis, N.I., Ryu, Y., de Voogt, P., Bramness, J. and Thomas, K., 2016. Comparison of pharmaceutical, illicit drug, alcohol, nicotine and caffeine levels in wastewater with sale, seizure and consumption data for 8 European cities. *Bmc Public Health,* 16.
[4] Ryu, Y., Reid, M.J. and Thomas, K.V., 2015. Liquid chromatography-high resolution mass spectrometry with immunoaffinity clean-up for the determination of the oxidative stress biomarker 8-iso-prostaglandin F2alpha in wastewater. *Journal of Chromatography A,* 1409, pp. 146-151.
[5] Choi, P.M., O'Brien, J.W., Li, J.Y., Jiang, G.M., Thomas, K.V. and Mueller, J.F., 2018. Population histamine burden assessed using wastewater-based epidemiology: The association of 1,4-methylimidazole acetic acid and fexofenadine. *Environment International,* 120, pp. 172-180.
[6] Stuveling, E.M., Hillege, H.L., Bakker, S.J.L., Gans, R.O.B., de Jong, P.E. and de Zeeuw, D., 2003. C-reactive protein is associated with renal function abnormalities in a non-diabetic population. *Kidney International,* 63(2), pp. 654-661.
[7] Postigo, C., Lopez de Alda, M. and Barcelo, D., 2011. Evaluation of drugs of abuse use and trends in a prison through wastewater analysis. *Environment International,* 37(1), pp. 49-55.
[8] Castrignano, E., Lubben, A. and Kasprzyk-Hordern, B., 2016. Enantiomeric profiling of chiral drug biomarkers in wastewater with the usage of chiral liquid chromatography coupled with tandem mass spectrometry. *Journal of Chromatography A,* 1438, pp. 84-99.
[9] Simon, R., Lemoine, J., Fonbonne, C., Jaffuel, A., Leonard, J.-F., Gautier, J.-C., Pasquier, O. and Salvador, A., 2014. Absolute quantification of podocin, a potential biomarker of glomerular injury in human urine, by liquid chromatography-multiple reaction monitoring cubed mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis,* 94, pp. 84-91.
[10] Simon, R., Passeron, S., Lemoine, J. and Salvador, A., 2014. Hydrophilic interaction liquid chromatography as second dimension in multidimensional chromatography with an anionic trapping strategy: Application to prostate-specific antigen quantification. *Journal of Chromatography A,* 1354, pp. 75-84.
[11] Ryu, Y., Gracia-Lor, E., Bade, R., Baz-Lomba, J.A., Bramness, J.G., Castiglioni, S., Castrignano, E., Causanilles, A., Covaci, A., de Voogt, P., Hernandez, F., Kasprzyk-Hordern, B., Kinyua, J., McCall, A.K., Ort, C., Plosz, B.G., Ramin, P., Rousis, N.I., Reid, M.J. and Thomas, K.V., 2016. Increased levels of the oxidative stress biomarker 8-iso-prostaglandin F-2 alpha in wastewater associated with tobacco use. *Scientific Reports,* 6.
[12] Castiglioni, S. and Gracia Lor, E., 2016. Target drug residues in wastewater. In: S. Castiglioni, ed. *Assessing illicit drugs in wastewater: advances in wastewater-based drug epidemiology.* 22 ed. Publications office of the European Union, Luxembourg: EMCDDA, pp. 35-45.
[13] Ort, C., van Nuijs, A.L.N., Berset, J.-D., Bijlsma, L., Castiglioni, S., Covaci, A., de Voogt, P., Emke, E., Fatta-Kassinos, D., Griffiths, P., Hernandez, F., Gonzalez-Marino, I., Grabic, R., Kasprzyk-Hordern, B., Mastroianni, N., Meierjohann, A., Nefau, T., Oestman, M., Pico, Y., Racamonde, I., Reid, M., Slobodnik, J., Terzic, S., Thomaidis, N. and Thomas, K.V., 2014. Spatial differences and temporal changes in illicit drug use in Europe quantified by wastewater analysis. *Addiction,* 109(8), pp. 1338-1352.
[14] Fortin, T., Salvador, A., Charrier, J.P., Lenz, C., Bettsworth, F., Lacoux, X., Choquet-Kastylevsky, G. and Lemoine, J., 2009. Multiple Reaction Monitoring Cubed for Protein Quantification at the Low

Nanogram/Milliliter Level in Nondepleted Human Serum. *Analytical Chemistry,* 81(22), pp. 9343-9352.
[15] Parker, C.E. and Borchers, C.H., 2014. Mass spectrometry based biomarker discovery, verification, and validation - Quality assurance and control of protein biomarker assays. *Molecular Oncology,* 8(4), pp. 840-858.
[16] Garovic, V.D., Craici, I.M., Wagner, S.J., White, W.M., Brost, B.C., Rose, C.H., Grande, J.P. and Barnidge, D.R., 2013. Mass spectrometry as a novel method for detection of podocyturia in pre-eclampsia. *Nephrology Dialysis Transplantation,* 28(6), pp. 1555-1561.
[17] Rodriguez-Suarez, E., Siwy, J., Zurbig, P. and Mischak, H., 2014. Urine as a source for clinical proteome analysis: From discovery to clinical application. *Biochimica Et Biophysica Acta-Proteins and Proteomics,* 1844(5), pp. 884-898.
[18] Barnidge, D.R., Goodmanson, M.K., Klee, G.G. and Muddiman, D.C., 2004. Absolute quantification of the model biomarker prostate-specific antigen in serum by LC-MS/MS using protein cleavage and isotope dilution mass spectrometry. *Journal of Proteome Research,* 3(3), pp. 644-652.
[19] Percy, A.J., Chambers, A.G., Yang, J., Hardie, D.B. and Borchers, C.H., 2014. Advances in multiplexed MRM-based protein biomarker quantitation toward clinical utility. *Biochimica Et Biophysica Acta-Proteins and Proteomics,* 1844(5), pp. 917-926.
[20] Shi, T.J., Fillmore, T.L., Gao, Y.Q., Zhao, R., He, J.T., Schepmoes, A.A., Nicora, C.D., Wu, C.C., Chambers, J.L., Moore, R.J., Kagan, J., Srivastava, S., Liu, A.Y., Rodland, K.D., Liu, T., Camp, D.G., Smith, R.D. and Qian, W.J., 2013. Long-Gradient Separations Coupled with Selected Reaction Monitoring for Highly Sensitive, Large Scale Targeted Protein Quantification in a Single Analysis. *Analytical Chemistry,* 85(19), pp. 9196-9203.
[21] Bijlsma, L., Botero-Coy, A.M., Rincon, R.J., Penuela, G.A. and Hernandez, F., 2016. Estimation of illicit drug use in the main cities of Colombia by means of urban wastewater analysis. *Science of the Total Environment,* 565, pp. 984-993.
[22] Gao, J.F., O'Brien, J., Du, P., Li, X.Q., Ort, C., Mueller, J.F. and Thai, P.K., 2016. Measuring selected PPCPs in wastewater to estimate the population in different cities in China. *Science of the Total Environment,* 568, pp. 164-170.
[23] Gracia-Lor, E., Castiglioni, S., Bade, R., Been, F., Castrignano, E., Covaci, A., Gonzalez-Marino, I., Hapeshi, E., Kasprzyk-Hordern, B., Kinyua, J., Lai, F.Y., Letzel, T., Lopardo, L., Meyer, M.R., O'Brien, J., Ramin, P., Rousis, N.I., Rydevik, A., Ryu, Y., Santos, M.M., Senta, I., Thomaidis, N.S., Veloutsou, S., Yang, Z.G., Zuccato, E. and Bijlsma, L., 2017. Measuring biomarkers in wastewater as a new source of epidemiological information: Current state and future perspectives. *Environment International,* 99, pp. 131-150.
[24] Sardh, E., Harper, P., Andersson, D.E.H. and Floderus, Y., 2009. Plasma porphobilinogen as a sensitive biomarker to monitor the clinical and therapeutic course of acute intermittent porphyria attacks. *European Journal of Internal Medicine,* 20(2), pp. 201-207.
[25] Buszewski, B. and Noga, S., 2012. Hydrophilic interaction liquid chromatography (HILIC)-a powerful separation technique. *Analytical and Bioanalytical Chemistry,* 402(1), pp. 231-247.
[26] Boersema, P.J., Mohammed, S. and Heck, A.J.R., 2008. Hydrophilic interaction liquid chromatography (HILIC) in proteomics. *Analytical and Bioanalytical Chemistry,* 391(1), pp. 151-159.
[27] Su, L., Cao, L., Zhou, R., Jiang, Z., Xiao, K., Kong, W., Wang, H., Deng, J., Wen, B., Tan, F., Zhang, Y. and Xie, L., 2013. Identification of Novel Biomarkers for Sepsis Prognosis via Urinary Proteomic Analysis Using iTRAQ Labeling and 2D-LC-MS/MS. *Plos One,* 8(1).
[28] Kinyua, J., Covaci, A., Maho, W., McCall, A.K., Neels, H. and Nuijs, A.L.N., 2015. Sewage-based epidemiology in monitoring the use of new psychoactive substances: Validation and application of an analytical method using LC-MS/MS. *Drug Testing and Analysis,* 7(9), pp. 812-818.
[29] Mardal, M., Kinyua, J., Ramin, P., Miserez, B., Van Nuijs, A.L.N., Covaci, A. and Meyer, M.R., 2017. Screening for illicit drugs in pooled human urine and urinated soil samples and studies on the stability of urinary excretion products of cocaine, MDMA, and MDEA in wastewater by hyphenated mass spectrometry techniques. *Drug Testing and Analysis,* 9(1), pp. 106-114.

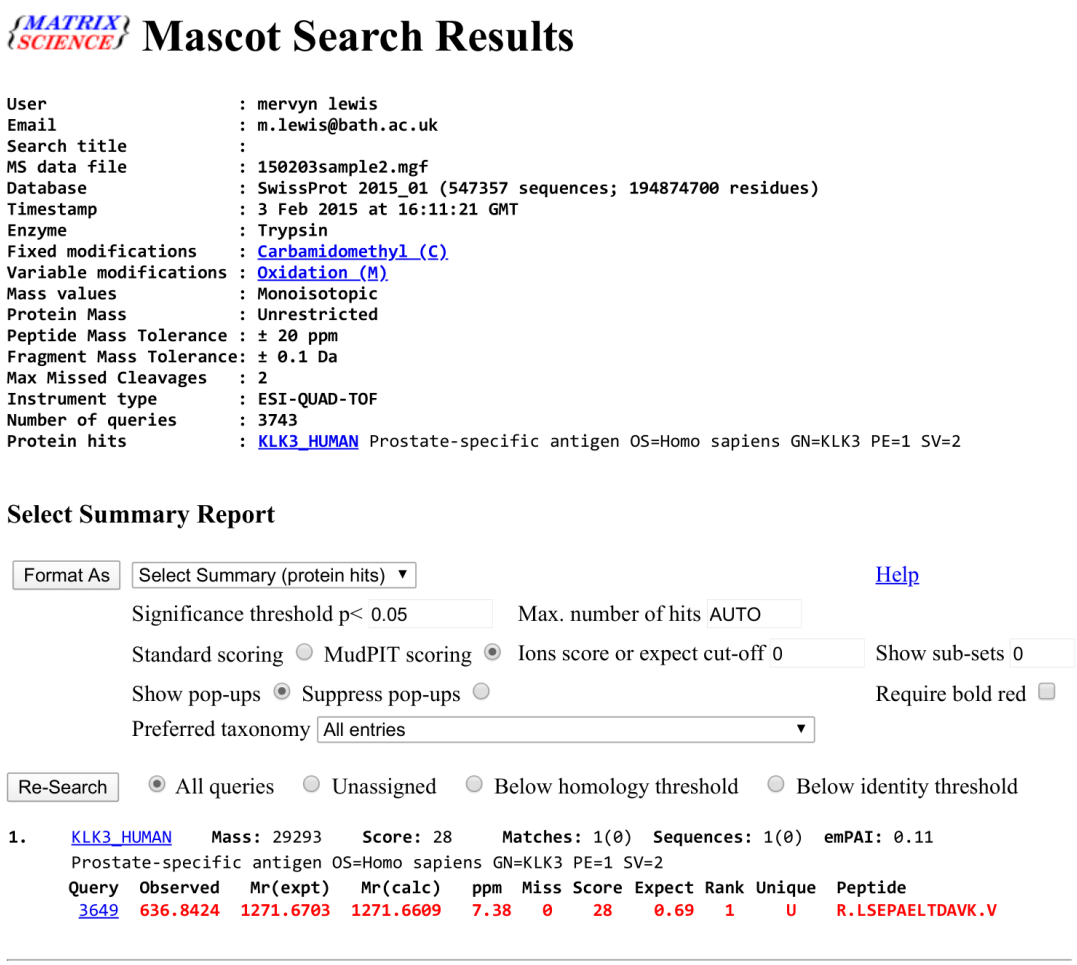
[30] Gao, T.-t., Du, P., Xu, Z.-q., Yang, J., Zhang, H.-f. and Li, X.-q., 2017. Optimization and Validation of the Analytical Method to Detect Common Illicit Drugs in Wastewater. *Huanjing Kexue,* 38(1), pp. 201-211.
[31] van Nuijs, A.L.N., Tarcomnicu, I., Bervoets, L., Blust, R., Jorens, P.G., Neels, H. and Covaci, A., 2009. Analysis of drugs of abuse in wastewater by hydrophilic interaction liquid chromatography-tandem mass spectrometry. *Analytical and Bioanalytical Chemistry,* 395(3), pp. 819-828.
[32] Kuzyk, M.A., Smith, D., Yang, J.C., Cross, T.J., Jackson, A.M., Hardie, D.B., Anderson, N.L. and Borchers, C.H., 2009. Multiple Reaction Monitoring-based, Multiplexed, Absolute Quantitation of 45 Proteins in Human Plasma. *Molecular & Cellular Proteomics,* 8(8), pp. 1860-1877.
[33] Yin, H.R., Zhang, L., Xie, L.Q., Huang, L.Y., Xu, Y., Cai, S.J., Yang, P.Y. and Lu, H.J., 2013. Hyperplex-MRM: A Hybrid Multiple Reaction Monitoring Method Using mTRAQ/iTRAQ Labeling for Multiplex Absolute Quantification of Human Colorectal Cancer Biomarker. *Journal of Proteome Research,* 12(9), pp. 3912-3919.
[34] Zauner, G., Koeleman, C.A.M., Deelder, A.M. and Wuhrer, M., 2010. Protein glycosylation analysis by HILIC-LC-MS of Proteinase K-generated N- and O-glycopeptides. *Journal of Separation Science,* 33(6-7), pp. 903-910.
[35] Gregorich, Z.R. and Ge, Y., 2014. Top-down proteomics in health and disease: Challenges and opportunities. *Proteomics,* 14(10), pp. 1195-1210.
[36] Pan, S., Aebersold, R., Chen, R., Rush, J., Goodlett, D.R., McIntosh, M.W., Zhang, J. and Brentnall, T.A., 2009. Mass Spectrometry Based Targeted Protein Quantification: Methods and Applications. *Journal of Proteome Research,* 8(2), pp. 787-797.
[37] Lowenthal, M.S., Liang, Y.X., Phinney, K.W. and Stein, S.E., 2014. Quantitative Bottom-Up Proteomics Depends on Digestion Conditions. *Analytical Chemistry,* 86(1), pp. 551-558.
[38] Walmsley, S.J., Rudnick, P.A., Liang, Y.X., Dong, Q., Stein, S.E. and Nesvizhskii, A.I., 2013. Comprehensive Analysis of Protein Digestion Using Six Trypsins Reveals the Origin of Trypsin As a Significant Source of Variability in Proteomics. *Journal of Proteome Research,* 12(12), pp. 5666-5680.
[39] Kilpatrick, E.L. and Bunk, D.M., 2009. Reference Measurement Procedure Development for C-Reactive Protein in Human Serum. *Analytical Chemistry,* 81(20), pp. 8610-8616.
[40] Vandermarliere, E., Mueller, M. and Martens, L., 2013. GETTING INTIMATE WITH TRYPSIN, THE LEADING PROTEASE IN PROTEOMICS. *Mass Spectrometry Reviews,* 32(6), pp. 453-465.
[41] Proc, J.L., Kuzyk, M.A., Hardie, D.B., Yang, J., Smith, D.S., Jackson, A.M., Parker, C.E. and Borchers, C.H., 2010. A Quantitative Study of the Effects of Chaotropic Agents, Surfactants, and Solvents on the Digestion Efficiency of Human Plasma Proteins by Trypsin. *Journal of Proteome Research,* 9(10), pp. 5422-5437.
[42] Chen, E.I., Cociorva, D., Norris, J.L. and Yates, J.R., III, 2007. Optimization of mass spectrometry-compatible surfactants for shotgun proteomics. *Journal of Proteome Research,* 6(7), pp. 2529-2538.
[43] Leon, I.R., Schwammle, V., Jensen, O.N. and Sprenger, R.R., 2013. Quantitative Assessment of In-solution Digestion Efficiency Identifies Optimal Protocols for Unbiased Protein Analysis. *Molecular & Cellular Proteomics,* 12(10), pp. 2992-3005.
[44] Burns, J.A., Butler, J.C., Moran, J. and Whitesides, G.M., 1991. SELECTIVE REDUCTION OF DISULFIDES BY TRIS(2-CARBOXYETHYL)PHOSPHINE. *Journal of Organic Chemistry,* 56(8), pp. 2648-2650.
[45] Burkhart, J.M., Schumbrutzki, C., Wortelkamp, S., Sickmann, A. and Zahedi, R.P., 2012. Systematic and quantitative comparison of digest efficiency and specificity reveals the impact of trypsin quality on MS-based proteomics. *Journal of Proteomics,* 75(4), pp. 1454-1462.
[46] Shuford, C.M., Sederoff, R.R., Chiang, V.L. and Muddiman, D.C., 2012. Peptide Production and Decay Rates Affect the Quantitative Accuracy of Protein Cleavage Isotope Dilution Mass Spectrometry (PC-IDMS). *Molecular & Cellular Proteomics,* 11(9), pp. 814-823.
[47] Adachi, J., Kumar, C., Zhang, Y., Olsen, J.V. and Mann, M., 2006. The human urinary proteome contains more than 1500 proteins, including a large proportion of membrane proteins. *Genome Biology,* 7(9).

[48] Anderson, L. and Hunter, C.L., 2006. Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins. *Molecular & Cellular Proteomics,* 5(4), pp. 573-588.
[49] Chiva, C., Ortega, M. and Sabido, E., 2014. Influence of the Digestion Technique, Protease, and Missed Cleavage Peptides in Protein Quantitation. *Journal of Proteome Research,* 13(9), pp. 3979-3986.
[50] Choi, Y.S., 2012. Reaching for the deep proteome: Recent nano liquid chromatography coupled with tandem mass spectrometry-based studies on the deep proteome. *Archives of Pharmacal Research,* 35(11), pp. 1861-1870.
[51] Davalieva, K., Kiprijanovska, S., Komina, S., Petrusevska, G., Zografska, N.C. and Polenakovic, M., 2015. Proteomics analysis of urine reveals acute phase response proteins as candidate diagnostic biomarkers for prostate cancer. *Proteome Science,* 13.
[52] Domanski, D., Percy, A.J., Yang, J., Chambers, A.G., Hill, J.S., Freue, G.V.C. and Borchers, C.H., 2012. MRM-based multiplexed quantitation of 67 putative cardiovascular disease biomarkers in human plasma. *Proteomics,* 12(8), pp. 1222-1243.
[53] Janecki, D.J., Bemis, K.G., Tegeler, T.J., Sanghani, P.C., Zhai, L., Hurley, T.D., Bosron, W.F. and Wang, M., 2007. A multiple reaction monitoring method for absolute quantification of the human liver alcohol dehydrogenase ADH1C1 isoenzyme. *Analytical Biochemistry,* 369(1), pp. 18-26.
[54] Kuhn, E., Wu, J., Karl, J., Liao, H., Zolg, W. and Guild, B., 2004. Quantification of C-reactive protein in the serum of patients with rheumatoid arthritis using multiple reaction monitoring mass spectrometry and C-13-labeled peptide standards. *Proteomics,* 4(4), pp. 1175-1186.
[55] Lesur, A., Varesio, E., Domon, B. and Hopfgartner, G., 2012. Peptides Quantification by Liquid Chromatography with Matrix-Assisted Laser Desorption/Ionization and Selected Reaction Monitoring Detection. *Journal of Proteome Research,* 11(10), pp. 4972-4982.
[56] Lin, S., Shaler, T.A. and Becker, C.H., 2006. Quantification of intermediate-abundance proteins in serum by multiple reaction monitoring mass spectrometry in a single-quadrupole ion trap. *Analytical Chemistry,* 78(16), pp. 5762-5767.
[57] Lin, Y., Liu, Y., Li, J.J., Zhao, Y., He, Q.Z., Han, W.J., Chen, P., Wang, X.C. and Liang, S.P., 2010. Evaluation and optimization of removal of an acid-insoluble surfactant for shotgun analysis of membrane proteome. *Electrophoresis,* 31(16), pp. 2705-2713.
[58] Lin, Y., Huo, L.J., Liu, Z.H., Li, J.L., Liu, Y., He, Q.Z., Wang, X.C. and Liang, S.P., 2013. Sodium Laurate, a Novel Protease- and Mass Spectrometry-Compatible Detergent for Mass Spectrometry-Based Membrane Proteomics. *Plos One,* 8(3).
[59] Loziuk, P.L., Wang, J., Li, Q., Sederoff, R.R., Chiang, V.L. and Muddiman, D.C., 2013. Understanding the Role of Proteolytic Digestion on Discovery and Targeted Proteomic Measurements Using Liquid Chromatography Tandem Mass Spectrometry and Design of Experiments. *Journal of Proteome Research,* 12(12), pp. 5820-5829.
[60] Masuda, T., Tomita, M. and Ishihama, Y., 2008. Phase transfer surfactant-aided trypsin digestion for membrane proteome analysis. *Journal of Proteome Research,* 7(2), pp. 731-740.
[61] Olkowicz, M., Rybakowska, I., Chlopicki, S. and Smolenski, R.T., 2015. Development and analytical comparison of microflow and nanoflow liquid chromatography/mass spectrometry procedures for quantification of cardiac troponin T in mouse hearts. *Talanta,* 131, pp. 510-520.
[62] Percy, A.J., Chambers, A.G., Yang, J., Jackson, A.M., Domanski, D., Burkhart, J., Sickmann, A. and Borchers, C.H., 2013. Method and platform standardization in MRM-based quantitative plasma proteomics. *Journal of Proteomics,* 95, pp. 66-76.
[63] Razavi, M., Frick, L.E., LaMarr, W.A., Pope, M.E., Miller, C.A., Anderson, N.L. and Pearson, T.W., 2012. High-Throughput SISCAPA Quantitation of Peptides from Human Plasma Digests by Ultrafast, Liquid Chromatography-Free Mass Spectrometry. *Journal of Proteome Research,* 11(12), pp. 5642-5649.
[64] Shi, T.J., Gao, Y.Q., Quek, S.I., Fillmore, T.L., Nicora, C.D., Su, D., Zhao, R., Kagan, J., Srivastava, S., Rodland, K.D., Liu, T., Smith, R.D., Chan, D.W., Camp, D.G., Liu, A.Y. and Qian, W.J., 2014. A Highly

Sensitive Targeted Mass Spectrometric Assay for Quantification of AGR2 Protein in Human Urine and Serum. *Journal of Proteome Research,* 13(2), pp. 875-882.
[65] Wehr, A.Y., Hwang, W.-T., Blair, I.A. and Yu, K.H., 2012. Relative Quantification of Serum Proteins from Pancreatic Ductal Adenocarcinoma Patients by Stable Isotope Dilution Liquid Chromatography-Mass Spectrometry. *Journal of Proteome Research,* 11(3), pp. 1749-1758.
[66] Whiteaker, J.R., Zhao, L., Schoenherr, R.M., Kennedy, J.J., Ivey, R.G. and Paulovich, A.G., 2017. Peptide Immunoaffinity Enrichment with Targeted Mass Spectrometry: Application to Quantification of ATM Kinase Phospho-Signaling. *Atm Kinase: Methods and Protocols,* 1599, pp. 197-213.
[67] Young, B.L., Mlamla, Z., Gqamana, P.P., Smit, S., Roberts, T., Peter, J., Theron, G., Goyender, U., Dheda, K. and Blackburn, J., 2014. The identification of tuberculosis biomarkers in human urine samples. *European Respiratory Journal,* 43(6), pp. 1719-1729.
[68] Gonzalez-Marino, I., Baz-Lomba, J.A., Alygizakis, N.A., Andres-Costa, M.J., Bade, R., Barron, L.P., Been, F., Berset, J.D., Bijlsma, L., Bodik, I., Brenner, A., Brock, A.L., Burgard, D.A., Castrignano, E., Christophoridis, C.E., Covaci, A., de Voogt, P., Devault, D.A., Dias, M.J., Emke, E., Fatta-Kassinos, D., Fedorova, G., Fytianos, K., Gerber, C., Grabic, R., Gruner, S., Gunnar, T., Hapeshi, E., Heath, E., Helm, B., Hernandez, F., Kankaanpaa, A., Karolak, S., Kasprzyk-Hordern, B., Krizman-Matasic, I., Lai, F.Y., Lechowicz, W., Lopes, A., de Alda, M.L., Lopez-Garcia, E., Love, A.S.C., Mastroianni, N., McEneff, G.L., Montes, R., Munro, K., Nefau, T., Oberacher, H., O'Brien, J.W., Olafsdottir, K., Pico, Y., Plosz, B.G., Polesel, F., Postigo, C., Quintana, J.B., Ramin, P., Reid, M.J., Rice, J., Rodil, R., Senta, I., Simoes, S.M., Sremacki, M.M., Styszko, K., Terzic, S., Thomaidis, N.S., Thomas, K.V., Tscharke, B., van Nuijs, A.L.N., Yargeau, V., Zuccato, E., Castiglioni, S. and Ort, C., 2020. Spatio-temporal assessment of illicit drug use at large scale: evidence from 7 years of international wastewater monitoring. *Addiction,* 115(1), pp. 109-120.
[69] Emke, E., Evans, S., Kasprzyk-Hordern, B. and de Voogt, P., 2014. Enantiomer profiling of high loads of amphetamine and MDMA in communal sewage: A Dutch perspective. *Science of the Total Environment,* 487, pp. 666-672.
[70] Petrie, B., Youdan, J., Barden, R. and Kasprzyk-Hordern, B., 2016. New Framework To Diagnose the Direct Disposal of Prescribed Drugs in Wastewater - A Case Study of the Antidepressant Fluoxetine. *Environmental Science & Technology,* 50(7), pp. 3781-3789.
[71] Du, P., Li, K.Y., Li, J., Xu, Z.Q., Fu, X.F., Yang, J., Zhang, H.F. and Li, X.Q., 2015. Methamphetamine and ketamine use in major Chinese cities, a nationwide reconnaissance through sewage-based epidemiology. *Water Research,* 84, pp. 76-84.
[72] Hammond, T.G., Moes, S., Youhanna, S., Jennings, P., Devuyst, O., Odermatt, A. and Jeno, P., 2016. Development and characterization of a pseudo multiple reaction monitoring method for the quantification of human uromodulin in urine. *Bioanalysis,* 8(12), pp. 1279-1296.
[73] Fortin, T., Salvador, A., Charrier, J.P., Lenz, C., Lacoux, X., Morla, A., Choquet-Kastylevsky, G. and Lemoine, J., 2009. Clinical Quantitation of Prostate-specific Antigen Biomarker in the Low Nanogram/Milliliter Range by Conventional Bore Liquid Chromatography-Tandem Mass Spectrometry (Multiple Reaction Monitoring) Coupling and Correlation with ELISA Tests. *Molecular & Cellular Proteomics,* 8(5), pp. 1006-1015.
[74] Kulasingam, V., Smith, C.R., Batruch, H., Buckler, A., Jeffery, D.A. and Diamandis, E.P., 2008. "Product ion monitoring" assay for prostate-specific antigen in serum using a linear ion-trap. *Journal of Proteome Research,* 7(2), pp. 640-647.
[75] Vegvari, A., Sjodin, K., Rezeli, M., Malm, J., Lilja, H., Laurell, T. and Marko-Varga, G., 2013. Identification of a Novel Proteoform of Prostate Specific Antigen (SNP-L132I) in Clinical Samples by Multiple Reaction Monitoring. *Molecular & Cellular Proteomics,* 12(10), pp. 2761-2773.
[76] Rice, J., Kannan, A.M., Castrignanò, E., Jagadeesan, K. and Kasprzyk-Hordern, B., 2020. Wastewater-based epidemiology combined with local prescription analysis as a tool for temporal monitoring of drug trends - a UK perspective. *[Submitted for publication]*.

[77] Centre for Disease Control, National Center for Emerging and Zoonotic Infectious Diseases and Division of Vector Born Diseases, 2019. *Hydration status* [Online]. www.cdc.gov/dengue/training/cme/ccm/Hydration%20Status_F.pdf: [Accessed 25-07-2019].
[78] Matheson, J., 2010. 'Average' Briton highlighted on UN World Statistics Day. Office for National Statistics, pp. 1-3.
[79] European Commission, 2002. *Implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results.* Official Journal of the European Communities.
[80] Slagter, M.H., Gooren, L.J.G., de Ronde, W., Soosaipillai, A., Scorilas, A., Giltay, E.J., Paliouras, M. and Diamandis, E.P., 2006. Serum and urine tissue kallikrein concentrations in male-to-female transsexuals treated with antiandrogens and estrogens. *Clinical Chemistry,* 52(7), pp. 1356-1365.
[81] Rice, J., Lubben, A. and Kasprzyk-Hordern, B., 2020. A multi-residue method by supercritical fluid chromatography coupled with tandem mass spectrometry for the analysis of chiral and non-chiral chemicals of emerging concern in environmental samples. *[Submitted for publication]*.
[82] Romero, F., Howard, J.M., Pairent, F.W. and Appert, H.E., 1972. 24 HOUR EXCRETION OF TRYPSIN AND TRYPSINOGEN IN URINE. *Annals of Surgery,* 176(2), pp. 154-&.

Supplementary information



MATRIX SCIENCE **Mascot Search Results**

```
User                  : mervyn lewis
Email                 : m.lewis@bath.ac.uk
Search title          :
MS data file          : 150203sample2.mgf
Database              : SwissProt 2015_01 (547357 sequences; 194874700 residues)
Timestamp             : 3 Feb 2015 at 16:11:21 GMT
Enzyme                : Trypsin
Fixed modifications   : Carbamidomethyl (C)
Variable modifications : Oxidation (M)
Mass values           : Monoisotopic
Protein Mass          : Unrestricted
Peptide Mass Tolerance : ± 20 ppm
Fragment Mass Tolerance: ± 0.1 Da
Max Missed Cleavages  : 2
Instrument type       : ESI-QUAD-TOF
Number of queries     : 3743
Protein hits          : KLK3_HUMAN Prostate-specific antigen OS=Homo sapiens GN=KLK3 PE=1 SV=2
```

Select Summary Report

Format As | Select Summary (protein hits) | Help

Significance threshold p< 0.05 | Max. number of hits AUTO

Standard scoring | MudPIT scoring | Ions score or expect cut-off 0 | Show sub-sets 0

Show pop-ups | Suppress pop-ups | Require bold red

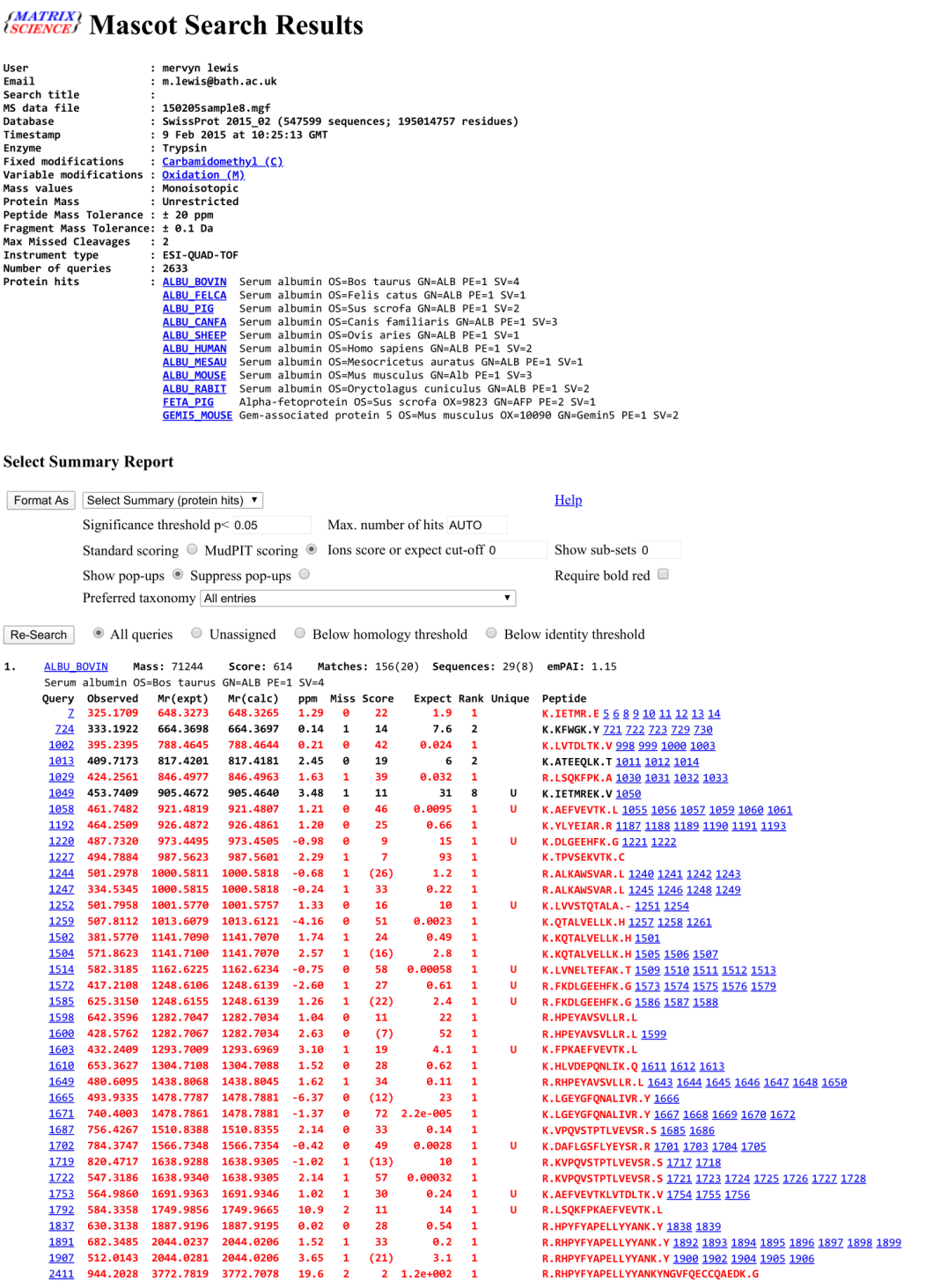
Preferred taxonomy: All entries

Re-Search | All queries | Unassigned | Below homology threshold | Below identity threshold

1. KLK3_HUMAN **Mass:** 29293 **Score:** 28 **Matches:** 1(0) **Sequences:** 1(0) **emPAI:** 0.11
Prostate-specific antigen OS=Homo sapiens GN=KLK3 PE=1 SV=2

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
3649	636.8424	1271.6703	1271.6609	7.38	0	28	0.69	1	U	R.LSEPAELTDAVK.V

Supplementary information (SI) figure 1. 30 nM concentration, enzyme only, PSA only digest



MATRIX SCIENCE **Mascot Search Results**

```
User                  : mervyn lewis
Email                 : m.lewis@bath.ac.uk
Search title          :
MS data file          : 150205sample8.mgf
Database              : SwissProt 2015_02 (547599 sequences; 195014757 residues)
Timestamp             : 9 Feb 2015 at 10:25:13 GMT
Enzyme                : Trypsin
Fixed modifications   : Carbamidomethyl (C)
Variable modifications : Oxidation (M)
Mass values           : Monoisotopic
Protein Mass          : Unrestricted
Peptide Mass Tolerance : ± 20 ppm
Fragment Mass Tolerance: ± 0.1 Da
Max Missed Cleavages  : 2
Instrument type       : ESI-QUAD-TOF
Number of queries     : 2633
Protein hits          : ALBU_BOVIN  Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4
                        ALBU_FELCA  Serum albumin OS=Felis catus GN=ALB PE=1 SV=1
                        ALBU_PIG    Serum albumin OS=Sus scrofa GN=ALB PE=1 SV=2
                        ALBU_CANFA  Serum albumin OS=Canis familiaris GN=ALB PE=1 SV=3
                        ALBU_SHEEP  Serum albumin OS=Ovis aries GN=ALB PE=1 SV=1
                        ALBU_HUMAN  Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2
                        ALBU_MESAU  Serum albumin OS=Mesocricetus auratus GN=ALB PE=1 SV=1
                        ALBU_MOUSE  Serum albumin OS=Mus musculus GN=Alb PE=1 SV=3
                        ALBU_RABIT  Serum albumin OS=Oryctolagus cuniculus GN=ALB PE=1 SV=2
                        FETA_PIG    Alpha-fetoprotein OS=Sus scrofa OX=9823 GN=AFP PE=2 SV=1
                        GEMI5_MOUSE Gem-associated protein 5 OS=Mus musculus OX=10090 GN=Gemin5 PE=1 SV=2
```

Select Summary Report

Format As: Select Summary (protein hits) — Help

Significance threshold p< 0.05 — Max. number of hits AUTO

Standard scoring / MudPIT scoring / Ions score or expect cut-off 0 — Show sub-sets 0

Show pop-ups / Suppress pop-ups — Require bold red

Preferred taxonomy: All entries

Re-Search — All queries / Unassigned / Below homology threshold / Below identity threshold

1. ALBU_BOVIN **Mass:** 71244 **Score:** 614 **Matches:** 156(20) **Sequences:** 29(8) **emPAI:** 1.15
Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
7	325.1709	648.3273	648.3265	1.29	0	22	1.9	1		K.IETMR.E 5 6 8 9 10 11 12 13 14
724	333.1922	664.3698	664.3697	0.14	1	14	7.6	2		K.KFWGK.Y 721 722 723 729 730
1002	395.2395	788.4645	788.4644	0.21	0	42	0.024	1		K.LVTDLTK.V 998 999 1000 1003
1013	409.7173	817.4201	817.4181	2.45	0	19	6	2		K.ATEEQLK.T 1011 1012 1014
1029	424.2561	846.4977	846.4963	1.63	1	39	0.032	1		R.LSQKFPK.A 1030 1031 1032 1033
1049	453.7409	905.4672	905.4640	3.48	1	11	31	8	U	K.IETMREK.V 1050
1058	461.7482	921.4819	921.4807	1.21	0	46	0.0095	1	U	K.AEFVEVTK.L 1055 1056 1057 1059 1060 1061
1192	464.2509	926.4872	926.4861	1.20	0	25	0.66	1		K.YLYEIAR.R 1187 1188 1189 1190 1191 1193
1220	487.7320	973.4495	973.4505	-0.98	0	9	15	1	U	K.DLGEEHFK.G 1221 1222
1227	494.7884	987.5623	987.5601	2.29	1	7	93	1		K.TPVSEKVTK.C
1244	501.2978	1000.5811	1000.5818	-0.68	1	(26)	1.2	1		R.ALKAWSVAR.L 1240 1241 1242 1243
1247	334.5345	1000.5815	1000.5818	-0.24	1	33	0.22	1		R.ALKAWSVAR.L 1245 1246 1248 1249
1252	501.7958	1001.5770	1001.5757	1.33	0	16	10	1	U	K.LVVSTQTALA.- 1251 1254
1259	507.8112	1013.6079	1013.6121	-4.16	0	51	0.0023	1		K.QTALVELLK.H 1257 1258 1261
1502	381.5770	1141.7090	1141.7070	1.74	1	24	0.49	1		K.KQTALVELLK.H 1501
1504	571.8623	1141.7100	1141.7070	2.57	1	(16)	2.8	1		K.KQTALVELLK.H 1505 1506 1507
1514	582.3185	1162.6225	1162.6234	-0.75	0	58	0.00058	1	U	K.LVNELTEFAK.T 1509 1510 1511 1512 1513
1572	417.2108	1248.6106	1248.6139	-2.60	1	27	0.61	1	U	R.FKDLGEEHFK.G 1573 1574 1575 1576 1579
1585	625.3150	1248.6155	1248.6139	1.26	1	(22)	2.4	1	U	R.FKDLGEEHFK.G 1586 1587 1588
1598	642.3596	1282.7047	1282.7034	1.04	0	11	22	1		R.HPEYAVSVLLR.L
1600	428.5762	1282.7067	1282.7034	2.63	0	(7)	52	1		R.HPEYAVSVLLR.L 1599
1603	432.2409	1293.7009	1293.6969	3.10	1	19	4.1	1	U	K.FPKAEFVEVTK.L
1610	653.3627	1304.7108	1304.7088	1.52	0	28	0.62	1		K.HLVDEPQNLIK.Q 1611 1612 1613
1649	480.6095	1438.8068	1438.8045	1.62	1	34	0.11	1		R.RHPEYAVSVLLR.L 1643 1644 1645 1646 1647 1648 1650
1665	493.9335	1478.7787	1478.7881	-6.37	0	(12)	23	1		K.LGEYGFQNALIVR.Y 1666
1671	740.4003	1478.7861	1478.7881	-1.37	0	72	2.2e-005	1		K.LGEYGFQNALIVR.Y 1667 1668 1669 1670 1672
1687	756.4267	1510.8388	1510.8355	2.14	0	33	0.14	1		K.VPQVSTPTLVEVSR.S 1685 1686
1702	784.3747	1566.7348	1566.7354	-0.42	0	49	0.0028	1	U	K.DAFLGSFLYEYSR.R 1701 1703 1704 1705
1719	820.4717	1638.9288	1638.9305	-1.02	1	(13)	10	1		R.KVPQVSTPTLVEVSR.S 1717 1718
1722	547.3186	1638.9340	1638.9305	2.14	1	57	0.00032	1		R.KVPQVSTPTLVEVSR.S 1721 1723 1724 1725 1726 1727 1728
1753	564.9860	1691.9363	1691.9346	1.02	1	30	0.24	1	U	K.AEFVEVTKLVTDLTK.V 1754 1755 1756
1792	584.3358	1749.9856	1749.9665	10.9	2	11	14	1	U	R.LSQKFPKAEFVEVTK.L
1837	630.3138	1887.9196	1887.9195	0.02	0	28	0.54	1		R.HPYFYAPELLYYANK.Y 1838 1839
1891	682.3485	2044.0237	2044.0206	1.52	1	33	0.2	1		R.RHPYFYAPELLYYANK.Y 1892 1893 1894 1895 1896 1897 1898 1899
1907	512.0143	2044.0281	2044.0206	3.65	1	(21)	3.1	1		R.RHPYFYAPELLYYANK.Y 1900 1902 1904 1905 1906
2411	944.2028	3772.7819	3772.7078	19.6	2	2	1.2e+002	1		R.RHPYFYAPELLYYANKYNGVFQECCQAEDK.G

Supplementary information (SI) figure 2. 606 nM concentration, enzyme only, BSA only digest 1

Mascot Search Results

```
User                  : mervyn lewis
Email                 : m.lewis@bath.ac.uk
Search title          :
MS data file          : 150205sample9.mgf
Database              : SwissProt 2015_02 (547599 sequences; 195014757 residues)
Timestamp             : 9 Feb 2015 at 10:33:14 GMT
Enzyme                : Trypsin
Fixed modifications   : Carbamidomethyl (C)
Variable modifications : Oxidation (M)
Mass values           : Monoisotopic
Protein Mass          : Unrestricted
Peptide Mass Tolerance : ± 20 ppm
Fragment Mass Tolerance: ± 0.1 Da
Max Missed Cleavages  : 2
Instrument type       : ESI-QUAD-TOF
Number of queries     : 2660
Protein hits          : ALBU_BOVIN Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4
                        ALBU_FELCA Serum albumin OS=Felis catus GN=ALB PE=1 SV=1
                        ALBU_CANFA Serum albumin OS=Canis familiaris GN=ALB PE=1 SV=3
                        ALBU_SHEEP Serum albumin OS=Ovis aries GN=ALB PE=1 SV=1
                        ALBU_MESAU Serum albumin OS=Mesocricetus auratus GN=ALB PE=1 SV=1
                        ALBU_MOUSE Serum albumin OS=Mus musculus GN=Alb PE=1 SV=3
                        FETA_PIG   Alpha-fetoprotein OS=Sus scrofa OX=9823 GN=AFP PE=2 SV=1
```

Select Summary Report

Format As: Select Summary (protein hits) — Help

Significance threshold p< 0.05 — Max. number of hits AUTO

Standard scoring / MudPIT scoring — Ions score or expect cut-off 0 — Show sub-sets 0

Show pop-ups / Suppress pop-ups — Require bold red

Preferred taxonomy: All entries

Re-Search — All queries / Unassigned / Below homology threshold / Below identity threshold

1. ALBU_BOVIN **Mass:** 71244 **Score:** 701 **Matches:** 166(24) **Sequences:** 28(9) **emPAI:** 1.35
Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
10	325.1712	648.3278	648.3265	1.97	0	18	5.3	1	U	K.IETMR.E 7 8 9 11 12 13 14 15 16
720	333.1920	664.3695	664.3697	-0.22	1	30	0.22	1		K.KFWGK.Y 715 717 718 719 722 723 724
753	345.1912	688.3678	688.3656	3.10	0	14	19	5		K.AWSVAR.L 745
1009	395.2394	788.4642	788.4644	-0.23	0	40	0.032	1	U	K.LVTDLTK.V 1005 1006 1007 1008 1010
1020	409.7156	817.4167	817.4181	-1.77	0	25	1.5	1		K.ATEEQLK.T 1016 1017 1018 1019 1021
1036	424.2559	846.4973	846.4963	1.17	1	38	0.045	1		R.LSQKFPK.A 1035 1037 1038 1039
1051	453.7397	905.4648	905.4640	0.88	1	12	25	1	U	K.IETMREK.V 1052 1053 1054
1064	461.7468	921.4791	921.4807	-1.74	0	44	0.014	1	U	K.AEFVEVTK.L 1065 1066 1067 1068 1069 1070
1146	464.2495	926.4844	926.4861	-1.84	0	26	0.51	1		K.YLYEIAR.R 1141 1142 1143 1144 1145 1147 1148
1185	494.7855	987.5565	987.5601	-3.61	1	6	1.2e+002	4		K.TPVSEKVTK.C 1184
1204	501.2985	1000.5824	1000.5818	0.68	1	(16)	12	4		R.ALKAWSVAR.L 1201 1202 1203
1205	334.5351	1000.5834	1000.5818	1.65	1	30	0.48	1		R.ALKAWSVAR.L 1206 1207 1208 1209 1210
1214	501.7950	1001.5754	1001.5757	-0.35	0	21	3.1	1	U	K.LVVSTQTALA.- 1211 1212
1218	507.8136	1013.6126	1013.6121	0.55	0	36	0.071	1		K.QTALVELLK.H 1216 1217 1220
1488	381.5759	1141.7059	1141.7070	-0.96	1	(16)	3	1		K.KQTALVELLK.H 1485 1486 1487
1491	571.8608	1141.7071	1141.7070	0.08	1	39	0.017	1		K.KQTALVELLK.H 1489 1490 1492
1497	582.3194	1162.6242	1162.6234	0.74	0	59	0.00059	1	U	K.LVNELTEFAK.T 1493 1494 1495 1496 1498
1551	625.3139	1248.6132	1248.6139	-0.57	1	(16)	8.8	2	U	R.FKDLGEEHFK.G 1549 1550 1552
1554	417.2117	1248.6132	1248.6139	-0.52	1	21	2.8	1	U	R.FKDLGEEHFK.G 1553 1556 1557 1558
1567	642.3610	1282.7075	1282.7034	3.23	0	(13)	13	1		R.HPEYAVSVLLR.L
1569	428.5766	1282.7079	1282.7034	3.56	0	18	4.1	1		R.HPEYAVSVLLR.L
1573	432.2366	1293.6880	1293.6969	-6.85	1	27	0.67	1	U	K.FPKAEFVEVTK.L 1572
1584	653.3601	1304.7057	1304.7088	-2.38	0	27	0.85	1		K.HLVDEPQNLIK.Q 1581 1582 1583
1625	480.6087	1438.8041	1438.8045	-0.22	1	45	0.0073	1		R.RHPEYAVSVLLR.L 1621 1622 1623 1624 1626 1627 1628
1640	493.9347	1478.7822	1478.7881	-4.02	0	(12)	26	1		K.LGEYGFQNALIVR.Y 1639
1645	740.4018	1478.7891	1478.7881	0.62	0	91	3e-007	1		K.LGEYGFQNALIVR.Y 1641 1642 1643 1644 1646
1660	756.4249	1510.8353	1510.8355	-0.13	0	12	16	1		K.VPQVSTPTLVEVSR.S 1658 1659 1661
1683	784.3737	1566.7328	1566.7354	-1.70	0	54	0.00082	1	U	K.DAFLGSFLYEYSR.R 1679 1680 1681 1682
1692	820.4719	1638.9292	1638.9305	-0.78	1	(17)	4	1		R.KVPQVSTPTLVEVSR.S 1694 1695
1696	547.3182	1638.9328	1638.9305	1.42	1	68	3e-005	1		R.KVPQVSTPTLVEVSR.S 1697 1698 1699 1700 1701 1702 1703
1730	846.9734	1691.9322	1691.9346	-1.41	1	(15)	9.5	1	U	K.AEFVEVTKLVTDLTK.V
1732	564.9889	1691.9449	1691.9346	6.14	1	30	0.19	1	U	K.AEFVEVTKLVTDLTK.V 1728 1731
1759	438.4998	1749.9701	1749.9665	2.06	2	12	15	1	U	R.LSQKFPKAEFVEVTK.L
1565	630.3143	1887.9212	1887.9195	0.87	0	38	0.16	1		R.HPYFYAPELLYYANK.Y 1798 1799 1800
1860	512.0113	2044.0160	2044.0206	-2.29	1	(24)	1.6	1		R.RHPYFYAPELLYYANK.Y 1855 1856 1857 1858 1859
1868	682.3472	2044.0198	2044.0206	-0.39	1	26	0.9	1		R.RHPYFYAPELLYYANK.Y 1861 1862 1863 1864 1865 1867 1869 1870

Supplementary information (SI) figure 3. 606 nM concentration, enzyme only, BSA only digest 2

Mascot Search Results

User : mervyn lewis
Email : m.lewis@bath.ac.uk
Search title :
MS data file : 150205sample5.mgf
Database : SwissProt 2015_01 (547357 sequences; 194874700 residues)
Timestamp : 5 Feb 2015 at 14:27:30 GMT
Enzyme : Trypsin
Fixed modifications : Carbamidomethyl (C)
Variable modifications : Oxidation (M)
Mass values : Monoisotopic
Protein Mass : Unrestricted
Peptide Mass Tolerance : ± 20 ppm
Fragment Mass Tolerance: ± 0.1 Da
Max Missed Cleavages : 2
Instrument type : ESI-QUAD-TOF
Number of queries : 1853
Protein hits : KLK3_HUMAN Prostate-specific antigen OS=Homo sapiens GN=KLK3 PE=1 SV=2

Select Summary Report

Format As: Select Summary (protein hits) — Help
Significance threshold p< 0.05 — Max. number of hits AUTO
Standard scoring / MudPIT scoring / Ions score or expect cut-off 0 — Show sub-sets 0
Show pop-ups / Suppress pop-ups — Require bold red
Preferred taxonomy: All entries
Re-Search — All queries / Unassigned / Below homology threshold / Below identity threshold

1. KLK3_HUMAN **Mass:** 29293 **Score:** 111 **Matches:** 19(3) **Sequences:** 5(1) **emPAI:** 0.11
Prostate-specific antigen OS=Homo sapiens GN=KLK3 PE=1 SV=2

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
984	379.2548	756.4950	756.4858	12.2	0	19	3.3	1	U	K.SVILLGR.H 981 982 983
1656	578.8219	1155.6293	1155.6288	0.44	1	18	6.3	1	U	K.WIKDTIVANP.- 1657 1658 1659
1751	636.8364	1271.6583	1271.6609	-2.01	0	72	2.6e-005	1	U	R.LSEPAELTDAVK.V 1750 1752 1753 1754 1755 1756 1757
1760	642.8676	1283.7206	1283.7238	-2.45	2	8	61	1	U	R.KWIKDTIVANP.-
1764	428.9147	1283.7222	1283.7238	-1.18	2	(2)	2.5e+002	1	U	R.KWIKDTIVANP.-
1801	469.9214	1406.7424	1406.7419	0.38	0	25	1.2	1	U	K.HSQPWQVLVASR.G

Supplementary information (SI) figure 4. 303 nM concentration, enzyme only, PSA only digest 1

Mascot Search Results

User : mervyn lewis
Email : m.lewis@bath.ac.uk
Search title :
MS data file : 150205sample6.mgf
Database : SwissProt 2015_01 (547357 sequences; 194874700 residues)
Timestamp : 5 Feb 2015 at 15:24:12 GMT
Enzyme : Trypsin
Fixed modifications : Carbamidomethyl (C)
Variable modifications : Oxidation (M)
Mass values : Monoisotopic
Protein Mass : Unrestricted
Peptide Mass Tolerance : ± 20 ppm
Fragment Mass Tolerance: ± 0.1 Da
Max Missed Cleavages : 2
Instrument type : ESI-QUAD-TOF
Number of queries : 1728
Protein hits : KLK3_HUMAN Prostate-specific antigen OS=Homo sapiens GN=KLK3 PE=1 SV=2
MUTL_SHEFN DNA mismatch repair protein MutL OS=Shewanella frigidimarina (strain NCIMB 400) OX=318167 GN=mutL PE=3 SV=1
HLR1_YEAST Protein HLR1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) OX=559292 GN=HLR1 PE=1 SV=1
RL4_THEAC 50S ribosomal protein L4 OS=Thermoplasma acidophilum (strain ATCC 25905 / DSM 1728 / JCM 9062 / NBRC 15155 / AMRC

Select Summary Report

Format As: Select Summary (protein hits) — Help
Significance threshold p< 0.05 — Max. number of hits AUTO
Standard scoring / MudPIT scoring / Ions score or expect cut-off 0 — Show sub-sets 0
Show pop-ups / Suppress pop-ups — Require bold red
Preferred taxonomy: All entries
Re-Search — All queries / Unassigned / Below homology threshold / Below identity threshold

1. KLK3_HUMAN **Mass:** 29293 **Score:** 67 **Matches:** 20(2) **Sequences:** 5(1) **emPAI:** 0.54
Prostate-specific antigen OS=Homo sapiens GN=KLK3 PE=1 SV=2

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1010	379.2502	756.4858	756.4858	0.02	0	36	0.093	1	U	K.SVILLGR.H 1006 1007 1008 1009
1655	578.8270	1155.6394	1155.6288	9.15	1	31	0.27	1	U	K.WIKDTIVANP.- 1652 1653 1654
1690	636.8382	1271.6618	1271.6609	0.74	0	48	0.0063	1	U	R.LSEPAELTDAVK.V 1686 1687 1688 1689 1691
1696	642.8688	1283.7230	1283.7238	-0.59	2	21	2.9	1	U	R.KWIKDTIVANP.- 1697
1701	469.9239	1406.7497	1406.7419	5.59	0	5	1.4e+002	1	U	K.HSQPWQVLVASR.G 1702 1703

Supplementary information (SI) figure 5. 303 nM concentration, enzyme only, PSA only digest 2

Mascot Search Results

```
User                    : mervyn lewis
Email                   : m.lewis@bath.ac.uk
Search title            :
MS data file            : 150204sample6.mgf
Database                : SwissProt 2015_01 (547357 sequences; 194874700 residues)
Timestamp               : 4 Feb 2015 at 16:28:50 GMT
Enzyme                  : Trypsin
Fixed modifications     : Carbamidomethyl (C)
Variable modifications  : Oxidation (M)
Mass values             : Monoisotopic
Protein Mass            : Unrestricted
Peptide Mass Tolerance  : ± 20 ppm
Fragment Mass Tolerance: ± 0.1 Da
Max Missed Cleavages    : 2
Instrument type         : ESI-QUAD-TOF
Number of queries       : 3006
Protein hits            : ALBU_BOVIN  Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4
                          ALBU_FELCA  Serum albumin OS=Felis catus GN=ALB PE=1 SV=1
                          ALBU_CANFA  Serum albumin OS=Canis familiaris GN=ALB PE=1 SV=3
                          ALBU_SHEEP  Serum albumin OS=Ovis aries GN=ALB PE=1 SV=1
                          ALBU_MOUSE  Serum albumin OS=Mus musculus GN=Alb PE=1 SV=3
                          ALBU_MESAU  Serum albumin OS=Mesocricetus auratus GN=ALB PE=1 SV=1
                          ALBU_MACFA  Serum albumin OS=Macaca fascicularis GN=ALB PE=2 SV=1
                          ALBU_HUMAN  Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2
                          ALBU_RABIT  Serum albumin OS=Oryctolagus cuniculus GN=ALB PE=1 SV=2
                          TRYP_PIG    Trypsin OS=Sus scrofa PE=1 SV=1
                          K2C6B_HUMAN Keratin, type II cytoskeletal 6B OS=Homo sapiens GN=KRT6B PE=1 SV=5
                          K2C6A_RAT   Keratin, type II cytoskeletal 6A OS=Rattus norvegicus GN=Krt6a PE=1 SV=1
                          K2C1_HUMAN  Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6
                          K1C9_HUMAN  Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3
                          ALBU_EQUAS  Serum albumin OS=Equus asinus GN=ALB PE=1 SV=1
                          ALBU_HORSE  Serum albumin OS=Equus caballus GN=ALB PE=1 SV=1
                          K22E_HUMAN  Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2
                          K1C10_HUMAN Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6
```

Select Summary Report

Format As [Select Summary (protein hits)] Help

Significance threshold p< 0.05 Max. number of hits AUTO

Standard scoring / MudPIT scoring / Ions score or expect cut-off 0 Show sub-sets 0

Show pop-ups / Suppress pop-ups Require bold red

Preferred taxonomy [All entries]

Re-Search All queries / Unassigned / Below homology threshold / Below identity threshold

1. ALBU_BOVIN **Mass:** 71244 **Score:** 894 **Matches:** 221(28) **Sequences:** 53(15) **emPAI:** 3.23
Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1000	333.1915	664.3685	664.3697	-1.74	1	21	1.5	1		K.KFWGK.Y 992 997 999 1002 1003 1004
1324	379.7152	757.4158	757.4156	0.22	0	33	0.18	1		K.GACLLPK.T 1325 1326 1327 1328 1329 1330 1331
1568	395.2399	788.4652	788.4644	1.03	0	40	0.038	1		K.LVTDLTK.V 1563 1564 1565 1566 1567
1607	409.7176	817.4207	817.4181	3.10	0	28	0.94	1		K.ATEEQLK.T 1608 1609
1677	424.2556	846.4966	846.4963	0.36	1	17	5.4	1		R.LSQKFPK.A 1675
1715	443.7115	885.4085	885.4080	0.64	0	34	0.056	1		K.DDSPDLPK.L 1714 1716 1717
1752	449.7442	897.4739	897.4742	-0.40	0	26	0.43	1		R.LCVLHEK.T 1753 1754 1755
1758	300.1657	897.4753	897.4742	1.24	0	(5)	67	1		R.LCVLHEK.T 1756
1853	461.7466	921.4787	921.4807	-2.26	0	30	0.32	1	U	K.AEFVEVTK.L 1852 1854 1855
1926	464.2507	926.4868	926.4861	0.70	0	30	0.19	1		K.YLYEIAR.R 1927 1928 1929 1930 1931
1961	487.7330	973.4515	973.4505	1.04	0	12	8.4	1		K.DLGEEHFK.G 1962
2003	501.7964	1001.5783	1001.5757	2.53	0	15	12	1	U	K.LVVSTQTALA.- 2004 2005 2006
2010	507.8128	1013.6111	1013.6121	-0.98	0	55	0.00091	1		K.QTALVELLK.H 2012 2013 2014
2281	534.7241	1067.4337	1067.4342	-0.51	0	37	0.0065	1	U	K.QNCDQFEK.L 2274 2276 2277 2279 2280
2285	358.1746	1071.5020	1071.5019	0.15	0	(6)	31	1	U	K.SHCIAEVEK.D 2287 2288
2292	536.7584	1071.5022	1071.5019	0.30	0	11	11	1	U	K.SHCIAEVEK.D 2289 2290 2291
2310	554.2596	1106.5047	1106.5066	-1.77	0	49	0.002	1		K.EACFAVEGPK.L 2311
2346	569.7538	1137.4931	1137.4907	2.11	0	29	0.11	1		K.CCTESLVNR.R 2343 2344 2345
2349	381.5765	1141.7077	1141.7070	0.62	1	(8)	22	1		K.KQTALVELLK.H 2347
2355	571.8625	1141.7105	1141.7070	3.06	1	45	0.0038	1		K.KQTALVELLK.H 2352 2353 2354
2375	582.3205	1162.6264	1162.6234	2.62	0	53	0.0021	1	U	K.LVNELTEFAK.T 2371 2372 2373 2374 2376
2510	428.5717	1282.6932	1282.7034	-7.89	0	(9)	33	1		R.HPEYAVSVLLR.L
2513	642.3589	1282.7033	1282.7034	-0.07	0	16	7.3	1		R.HPEYAVSVLLR.L 2511 2512
2514	431.2064	1290.5973	1290.5948	1.98	0	15	4.5	1		K.ECCDKPLLEK.S 2515

4/17/2020 Select Summary Report (../data/20150204/F001930.dat)

2517	646.3077	1290.6009	1290.5948	4.76	0	(14)	8.3	1		K.ECCDKPLLEK.S
2528	653.3607	1304.7068	1304.7088	-1.59	0	37	0.079	1		K.HLVDEPQNLIK.Q 2525 2526 2527 2529
2530	435.9116	1304.7130	1304.7088	3.19	0	(7)	71	1		K.HLVDEPQNLIK.Q 2531
2591	710.3494	1418.6843	1418.6864	-1.46	0	(20)	3	1		K.SLHTLFGDELCK.V 2589 2590 2592
2593	473.9023	1418.6852	1418.6864	-0.83	0	34	0.11	1		K.SLHTLFGDELCK.V 1941 2594 2595 2596 2597
2607	480.6085	1438.8036	1438.8045	-0.59	1	34	0.11	1		R.RHPEYAVSVLLR.L 2605 2606 2608 2609 2610
2616	722.3247	1442.6349	1442.6347	0.11	0	52	0.00054	1	U	K.YICDNQDTISSK.L 2617 2618 2619 2620 2621
2629	732.2979	1462.5812	1462.5817	-0.32	0	(22)	0.09	1	U	K.TCVADESHAGCEK.S 2628 2631
2632	488.5355	1462.5848	1462.5817	2.13	0	27	0.034	1	U	K.TCVADESHAGCEK.S 2633 2634 2635 2636
2663	740.4014	1478.7883	1478.7881	0.12	0	97	7.7e-008	1		K.LGEYGFQNALIVR.Y 2662 2664 2665 2666
2683	747.7646	1493.5147	1493.5109	2.59	0	16	0.023	1		R.ETYGDMADCCEK.Q 2682
2699	751.8096	1501.6047	1501.6065	-1.19	0	60	2e-005	1	U	K.EYEATLEECCAK.D 2700 2701 2702
2703	756.4246	1510.8347	1510.8355	-0.58	0	20	2.7	1		K.VPQVSTPTLVEVSR.S 2705
2727	511.5989	1531.7749	1531.7738	0.71	1	9	60	2		K.LKECCDKPLLEK.S 2729
2759	518.8901	1553.6485	1553.6457	1.84	0	20	0.37	1	U	K.DDPHACYSTVFDK.L 2036 2758 2760
2773	784.3748	1566.7350	1566.7354	-0.31	0	70	2.4e-005	1	U	K.DAFLGSFLYEYSR.R 2768 2769 2770 2771 2772
2776	526.2604	1575.7594	1575.7603	-0.57	0	20	3	1	U	K.LKPDPNTLCDEFK.A 2775 2777 2778 2779 2780
2784	788.8877	1575.7609	1575.7603	0.37	0	(9)	44	1	U	K.LKPDPNTLCDEFK.A
2819	547.3177	1638.9313	1638.9305	0.48	1	70	1.8e-005	1		R.KVPQVSTPTLVEVSR.S 2814 2815 2816 2817 2818
2854	580.9488	1739.8245	1739.8222	1.28	0	(9)	31	1		R.MPCTEDYLSLILNR.L 2853
2855	870.9197	1739.8248	1739.8222	1.49	0	16	6.9	1		R.MPCTEDYLSLILNR.L
2858	874.3551	1746.6957	1746.6978	-1.21	0	27	0.027	1		K.YNGVFQECCQAEDK.G 2857 2859 2860
2866	583.8919	1748.6538	1748.6553	-0.83	0	23	0.02	1		K.ECCHGDLLECADDR.A 2865 2867
2880	599.2842	1794.8308	1794.8247	3.42	1	10	20	1	U	K.DDPHACYSTVFDKLK.H 2881
2886	605.6177	1813.8312	1813.8226	4.75	1	13	9.6	1	U	R.LAKEYEATLEECCAK.D 2887
2895	940.9653	1879.9160	1879.9138	1.15	0	(8)	57	1	U	R.RPCFSALTPDETYVPK.A
2900	627.6470	1879.9191	1879.9138	2.80	0	17	6.9	1	U	R.RPCFSALTPDETYVPK.A 2897 2898 2899 2901 2902
2904	630.3136	1887.9191	1887.9195	-0.25	0	12	19	1		R.HPYFYAPELLYYANK.Y 2903
2908	634.6308	1900.8705	1900.8625	4.19	1	6	42	1	U	R.NECFLSHKDDSPDLPK.L
2912	636.6440	1906.9101	1906.9135	-1.78	0	24	1.3	1	U	K.LFTFHADICTLPDTEK.Q 2910 2911 2913
2919	643.2730	1926.7971	1926.7910	3.16	1	24	0.098	1	U	K.CCAADDKEACFAVEGPK.L 2918
2929	505.7474	2018.9603	2018.9619	-0.78	1	7	50	1	U	K.LKPDPNTLCDEFKADEK.K
2939	673.9946	2018.9619	2018.9619	-0.00	1	(6)	63	1	U	K.LKPDPNTLCDEFKADEK.K 2935
2944	682.3472	2044.0197	2044.0206	-0.47	1	24	1.4	1		R.RHPYFYAPELLYYANK.Y 2945
2951	529.2291	2112.8874	2112.8775	4.66	1	14	1.2	1		K.VHKECCHGDLLECADDR.A 2265 2950
2957	562.7407	2246.9337	2246.9354	-0.76	1	13	1.2	1		K.ECCHGDLLECADDRADLAK.Y 2959 2960
2970	820.0651	2457.1735	2457.1733	0.06	1	7	50	1	U	K.DAIPENLPPLTADFAEDKDVCK.N
2978	829.7095	2486.1067	2486.1028	1.56	1	6	26	1		K.YNGVFQECCQAEDKGACLLPK.I 2977 2979 2980
2984	831.4232	2491.2477	2491.2570	-3.71	0	23	2	1	U	K.GLVLIAFSQYLQQCPFDEHVK.L 2983 2985
2999	523.2390	2611.1588	2611.1577	0.43	2	0	73	1		K.VHKECCHGDLLECADDRADLAK.Y
3005	703.1445	3510.6860	3510.6647	6.07	2	11	19	1	U	K.SHCIAEVEKDAIPENLPPLTADFAEDKDVCK.N 3004

Supplementary information (SI) figure 6. 30 nM concentration, no surfactant, BSA only digest 1

Mascot Search Results

```
User                  : mervyn lewis
Email                 : m.lewis@bath.ac.uk
Search title          :
MS data file          : 150204sample7.mgf
Database              : SwissProt 2015_01 (547357 sequences; 194874700 residues)
Timestamp             : 5 Feb 2015 at 09:30:14 GMT
Enzyme                : Trypsin
Fixed modifications   : Carbamidomethyl (C)
Variable modifications : Oxidation (M)
Mass values           : Monoisotopic
Protein Mass          : Unrestricted
Peptide Mass Tolerance : ± 20 ppm
Fragment Mass Tolerance: ± 0.1 Da
Max Missed Cleavages  : 2
Instrument type       : ESI-QUAD-TOF
Number of queries     : 2711
Protein hits          : ALBU_BOVIN  Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4
                        ALBU_FELCA  Serum albumin OS=Felis catus GN=ALB PE=1 SV=1
                        ALBU_SHEEP  Serum albumin OS=Ovis aries GN=ALB PE=1 SV=1
                        ALBU_HUMAN  Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2
                        ALBU_MACFA  Serum albumin OS=Macaca fascicularis GN=ALB PE=2 SV=1
                        ALBU_CANFA  Serum albumin OS=Canis familiaris GN=ALB PE=1 SV=3
                        ALBU_MOUSE  Serum albumin OS=Mus musculus GN=Alb PE=1 SV=3
                        K2C1_HUMAN  Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6
                        TRYP_PIG    Trypsin OS=Sus scrofa PE=1 SV=1
                        ALBU_RABIT  Serum albumin OS=Oryctolagus cuniculus GN=ALB PE=1 SV=2
                        K1C10_HUMAN Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6
                        FETA_PIG    Alpha-fetoprotein OS=Sus scrofa OX=9823 GN=AFP PE=2 SV=1
```

Select Summary Report

Format As [Select Summary (protein hits)] Help

Significance threshold p< 0.05 Max. number of hits AUTO

Standard scoring ○ MudPIT scoring ◉ Ions score or expect cut-off 0 Show sub-sets 0

Show pop-ups ◉ Suppress pop-ups ○ Require bold red ☐

Preferred taxonomy [All entries]

Re-Search ◉ All queries ○ Unassigned ○ Below homology threshold ○ Below identity threshold

1. ALBU_BOVIN **Mass:** 71244 **Score:** 986 **Matches:** 220(37) **Sequences:** 51(15) **emPAI:** 3.04
Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
888	333.1913	664.3680	664.3697	-2.46	1	21	1.6	1		K.KFWGK.Y 878 881 884 886 887 890
959	345.1898	688.3651	688.3656	-0.85	0	12	30	10		K.AWSVAR.L
1189	379.7152	757.4159	757.4156	0.37	0	39	0.041	1		K.GACLLPK.I 1184 1185 1186 1187 1188 1190 1191
1400	395.2393	788.4641	788.4644	-0.35	0	37	0.068	1		K.LVTDLTK.V 1395 1396 1397 1398 1399
1442	409.7181	817.4217	817.4181	4.36	0	21	4.5	1		K.ATEEQLK.T 1441 1443
1500	424.2557	846.4968	846.4963	0.56	1	32	0.19	1		R.LSQKFPK.A 1499 1501 1502 1503
1536	443.7117	885.4088	885.4080	0.95	0	31	0.1	1		K.DDSPDLPK.L 1533 1534 1535
1567	300.1652	897.4736	897.4742	-0.65	0	(2)	1.1e+002	1		R.LCVLHEK.T
1570	449.7449	897.4753	897.4742	1.25	0	26	0.55	1		R.LCVLHEK.T 1568 1569 1571
1666	461.7474	921.4802	921.4807	-0.58	0	40	0.031	1	U	K.AEFVEVTK.L 1665 1667 1669 1670
1715	464.2504	926.4862	926.4861	0.08	0	28	0.34	1		K.YLYEIAR.R 1714 1716 1717 1718 1719
1745	487.7325	973.4505	973.4505	0.04	0	7	26	4		K.DLGEEHFK.G
1791	334.5346	1000.5821	1000.5818	0.31	1	11	33	1		R.ALKAWSVAR.L 1790
1797	501.7941	1001.5736	1001.5757	-2.15	0	34	0.16	1	U	K.LVVSTQTALA.- 1793 1794 1796
1802	507.8131	1013.6116	1013.6121	-0.45	0	38	0.048	1		K.QTALVELLK.H 1803 1804 1805
2068	534.7242	1067.4339	1067.4342	-0.32	0	33	0.017	1	U	K.QNCDQFEK.L 2064 2065 2066 2067 2069 2070 2071
2077	358.1746	1071.5019	1071.5019	-0.02	0	(1)	1.1e+002	1	U	K.SHCIAEVEK.D
2081	536.7594	1071.5043	1071.5019	2.27	0	18	2.3	1	U	K.SHCIAEVEK.D 2080 2082
2088	554.2600	1106.5055	1106.5066	-1.06	0	31	0.14	1		K.EACFAVEGPK.L 2089
2108	569.7538	1137.4931	1137.4907	2.16	0	42	0.0051	1		K.CCTESLVNR.R 2107 2109 2110
2113	381.5761	1141.7065	1141.7070	-0.42	1	(15)	4	1		K.KQTALVELLK.H 2112 2114
2118	571.8608	1141.7070	1141.7070	0.00	1	22	0.83	1		K.KQTALVELLK.H 2115 2116 2117
2137	582.3186	1162.6227	1162.6234	-0.55	0	50	0.004	1	U	K.LVNELTEFAK.T 2136 2138 2139 2140
2243	417.2046	1248.5918	1248.6139	-17.64	1	15	7.9	1		R.FKDLGEEHFK.G 2244
2248	625.3136	1248.6126	1248.6139	-0.99	1	(3)	1.6e+002	1		R.FKDLGEEHFK.G
2252	428.5739	1282.6997	1282.7034	-2.83	0	9	36	1		R.HPEYAVSVLLR.L
2256	646.3038	1290.5930	1290.5948	-1.42	0	(3)	76	1		K.ECCDKPLLEK.S
2261	431.2063	1290.5970	1290.5948	1.74	0	6	44	1		K.ECCDKPLLEK.S 2259 2260
2267	435.9090	1304.7051	1304.7088	-2.88	0	(10)	42	1		K.HLVDEPQNLIK.Q 2268
2272	653.3620	1304.7095	1304.7088	0.51	0	29	0.58	1		K.HLVDEPQNLIK.Q 2269 2270 2271

4/17/2020 Select Summary Report (../data/20150205/F001931.dat)

2310	473.9031	1418.6876	1418.6864	0.83	0	44	0.012	1		K.SLHTLFGDELCK.V 2311 2312 2313 2314 2315
2316	710.3531	1418.6916	1418.6864	3.68	0	(35)	0.1	1		K.SLHTLFGDELCK.V 2317 2318 2319
2330	480.6089	1438.8049	1438.8045	0.31	1	43	0.012	1		R.RHPEYAVSVLLR.L 2326 2327 2328 2329 2331
2334	722.3242	1442.6339	1442.6347	-0.61	0	90	9.3e-008	1	U	K.YICDNQDTISSK.L 2335 2336 2337 2338 2339
2353	732.2999	1462.5852	1462.5817	2.37	0	42	0.00097	1	U	K.TCVADESHAGCEK.S 2350 2351 2352
2357	488.5361	1462.5866	1462.5817	3.33	0	(36)	0.0049	1	U	K.TCVADESHAGCEK.S 2354 2355 2356 2358 2359
2388	740.3994	1478.7843	1478.7881	-2.62	0	80	3.8e-006	1	U	K.LGEYGFQNALIVR.Y 2387 2389 2390
2397	747.7583	1493.5021	1493.5109	-5.87	0	9	0.12	1		R.ETYGDMADCCEK.Q 2398
2418	751.8096	1501.6046	1501.6065	-1.23	0	60	1.7e-005	1	U	K.EYEATLEECCAK.D 2416 2417 2419
2420	756.4241	1510.8336	1510.8355	-1.30	0	22	1.6	1		K.VPQVSTPTLVEVSR.S 2422 2423
2446	511.5997	1531.7773	1531.7738	2.31	1	11	32	1		K.LKECCDKPLLEK.S 2448
2452	383.9518	1531.7782	1531.7738	2.85	1	(1)	3.7e+002	1		K.LKECCDKPLLEK.S
2475	777.8304	1553.6463	1553.6457	0.39	0	(11)	2.6	1	U	K.DDPHACYSTVFDK.L
2480	518.8906	1553.6501	1553.6457	2.84	0	23	0.17	1	U	K.DDPHACYSTVFDK.L 2477 2478 2479
2487	784.3738	1566.7331	1566.7354	-1.52	0	58	0.00033	1	U	K.DAFLGSFLYEYSR.R 2485 2486 2488
2491	788.8881	1575.7617	1575.7603	0.89	0	(24)	1.2	1	U	K.LKPDPNTLCDEFK.A
2498	526.2617	1575.7632	1575.7603	1.85	0	33	0.16	1	U	K.LKPDPNTLCDEFK.A 2493 2494 2495 2496 2497
2531	820.4719	1638.9292	1638.9305	-0.78	1	(30)	0.19	1		R.KVPQVSTPTLVEVSR.S 2529
2535	547.3176	1638.9311	1638.9305	0.35	1	66	4.9e-005	1		R.KVPQVSTPTLVEVSR.S 2532 2533 2534 2536 2537
2570	874.3546	1746.6947	1746.6978	-1.74	0	43	0.00062	1		K.YNGVFQECCQAEDK.G 2569 2571 2572
2579	583.8930	1748.6571	1748.6553	1.03	0	18	0.061	1		K.ECCHGDLLECADDR.A 2145 2578
2590	599.2822	1794.8249	1794.8247	0.11	1	6	39	1	U	K.DDPHACYSTVFDKLK.H 2591 2592
2598	605.6152	1813.8239	1813.8226	0.71	1	17	2.8	1	U	R.LAKEYEATLEECCAK.D 2597
2606	627.6471	1879.9195	1879.9138	2.99	0	20	3.7	1	U	R.RPCFSALTPDETYVPK.A 2601 2602 2603 2604 2605
2609	634.6274	1900.8604	1900.8625	-1.10	1	4	51	1	U	R.NECFLSHKDDSPDLPK.L
2617	636.6450	1906.9131	1906.9135	-0.20	0	20	3.4	1	U	K.LFTFHADICTLPDTEK.Q 2614 2615 2616
2255	643.2719	1926.7938	1926.7910	1.45	1	17	1.6	1	U	K.CCAADDKEACFAVEGPK.L 2621
2635	505.7471	2018.9592	2018.9619	-1.32	1	(4)	1.1e+002	1	U	K.LKPDPNTLCDEFKADEK.K
2643	673.9939	2018.9599	2018.9619	-0.99	1	11	19	1	U	K.LKPDPNTLCDEFKADEK.K 2638 2639 2641 2642
2645	682.3494	2044.0264	2044.0206	2.80	1	12	24	1		R.RHPYFYAPELLYYANK.Y 2644
2661	529.2284	2112.8844	2112.8775	3.25	1	14	1.2	1		K.VHKECCHGDLLECADDR.A 2658 2660
2674	562.7400	2246.9309	2246.9354	-2.04	1	7	4.7	1		K.ECCHGDLLECADDRADLAK.Y
2676	749.9881	2246.9425	2246.9354	3.13	1	(1)	29	1		K.ECCHGDLLECADDRADLAK.Y
2686	831.4293	2491.2660	2491.2570	3.60	0	20	3.5	1	U	K.GLVLIAFSQYLQQCPFDEHVK.L 2687 2688 2689
2707	523.2407	2611.1672	2611.1577	3.64	2	11	8	1		K.VHKECCHGDLLECADDRADLAK.Y

Supplementary information (SI) figure 7. 30 nM concentration, no surfactant, BSA only digest 2

Mascot Search Results

```
User                  : mervyn lewis
Email                 : m.lewis@bath.ac.uk
Search title          :
MS data file          : 150204sample4.mgf
Database              : SwissProt 2015_01 (547357 sequences; 194874700 residues)
Timestamp             : 4 Feb 2015 at 14:54:09 GMT
Enzyme                : Trypsin
Fixed modifications   : Carbamidomethyl (C)
Variable modifications : Oxidation (M)
Mass values           : Monoisotopic
Protein Mass          : Unrestricted
Peptide Mass Tolerance : ± 20 ppm
Fragment Mass Tolerance: ± 0.1 Da
Max Missed Cleavages  : 2
Instrument type       : ESI-QUAD-TOF
Number of queries     : 2557
Protein hits          : KLK3_HUMAN  Prostate-specific antigen OS=Homo sapiens GN=KLK3 PE=1 SV=2
                        TRYP_PIG    Trypsin OS=Sus scrofa PE=1 SV=1
                        K2C1_HUMAN  Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6
                        K22E_HUMAN  Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2
                        NPC2_HUMAN  Epididymal secretory protein E1 OS=Homo sapiens GN=NPC2 PE=1 SV=1
                        K1C10_HUMAN Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6
                        K22E_MOUSE  Keratin, type II cytoskeletal 2 epidermal OS=Mus musculus GN=Krt2 PE=1 SV=1
                        K1C9_HUMAN  Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3
```

Select Summary Report

Format As [Select Summary (protein hits)] Help

Significance threshold p< 0.05 Max. number of hits AUTO

Standard scoring / MudPIT scoring Ions score or expect cut-off 0 Show sub-sets 0

Show pop-ups / Suppress pop-ups Require bold red

Preferred taxonomy [All entries]

Re-Search All queries / Unassigned / Below homology threshold / Below identity threshold

1. KLK3_HUMAN **Mass:** 29293 **Score:** 88 **Matches:** 24(2) **Sequences:** 6(1) **emPAI:** 0.38
Prostate-specific antigen OS=Homo sapiens GN=KLK3 PE=1 SV=2

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1368	379.2498	756.4850	756.4858	-1.04	0	24	1.3	1	U	K.SVILLGR.H 1369 1370 1371
1817	435.7003	869.3860	869.3888	-3.17	0	7	18	1	U	K.FMLCAGR.W
2352	539.2572	1076.4998	1076.4961	3.45	0	24	0.81	1	U	R.IVGGWECEK.H 2349 2350 2351
2382	578.8246	1155.6346	1155.6288	5.04	1	12	22	1	U	K.WIKDTIVANP.- 2384
2485	636.8370	1271.6594	1271.6609	-1.19	0	45	0.014	1	U	R.LSEPAELTDAVK.V 2484 2486 2487 2489
2531	469.9211	1406.7414	1406.7419	-0.34	0	23	1.8	1	U	K.HSQPWQVLVASR.G 2526 2527 2528 2529 2530
2532	704.3801	1406.7456	1406.7419	2.65	0	(16)	10	1	U	K.HSQPWQVLVASR.G 2535

Supplementary information (SI) figure 8. 30 nM concentration, no surfactant, PSA only digest 1

Mascot Search Results

```
User                  : mervyn lewis
Email                 : m.lewis@bath.ac.uk
Search title          :
MS data file          : 150204sample10.mgf
Database              : SwissProt 2015_01 (547357 sequences; 194874700 residues)
Timestamp             : 5 Feb 2015 at 09:38:11 GMT
Enzyme                : Trypsin
Fixed modifications   : Carbamidomethyl (C)
Variable modifications : Oxidation (M)
Mass values           : Monoisotopic
Protein Mass          : Unrestricted
Peptide Mass Tolerance : ± 20 ppm
Fragment Mass Tolerance: ± 0.1 Da
Max Missed Cleavages  : 2
Instrument type       : ESI-QUAD-TOF
Number of queries     : 2100
Protein hits          : KLK3_HUMAN  Prostate-specific antigen OS=Homo sapiens GN=KLK3 PE=1 SV=2
                        TRYP_PIG    Trypsin OS=Sus scrofa PE=1 SV=1
                        K1C10_BOVIN Keratin, type I cytoskeletal 10 OS=Bos taurus OX=9913 GN=KRT10 PE=3 SV=1
                        K2C1_HUMAN  Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6
                        NPC2_HUMAN  Epididymal secretory protein E1 OS=Homo sapiens GN=NPC2 PE=1 SV=1
```

Select Summary Report

Format As [Select Summary (protein hits)] Help

Significance threshold p< 0.05 Max. number of hits AUTO

Standard scoring ○ MudPIT scoring ● Ions score or expect cut-off 0 Show sub-sets 0

Show pop-ups ● Suppress pop-ups ○ Require bold red ☐

Preferred taxonomy [All entries]

Re-Search ● All queries ○ Unassigned ○ Below homology threshold ○ Below identity threshold

1. KLK3_HUMAN **Mass:** 29293 **Score:** 94 **Matches:** 21(2) **Sequences:** 6(1) **emPAI:** 0.24
Prostate-specific antigen OS=Homo sapiens GN=KLK3 PE=1 SV=2

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1214	379.2506	756.4867	756.4858	1.30	0	30	0.35	1	U	K.SVILLGR.H 1215 1216 1217
1507	435.7029	869.3913	869.3888	2.94	0	6	24	1	U	K.FMLCAGR.W 1506
2007	539.2564	1076.4982	1076.4961	2.00	0	27	0.39	1	U	R.IVGGWECEK.H 2005 2006 2008
2022	578.8228	1155.6310	1155.6288	1.92	1	1	2.6e+002	1	U	K.WIKDTIVANP.-
2068	636.8386	1271.6627	1271.6609	1.46	0	43	0.02	1	U	R.LSEPAELTDAVK.V 2066 2067 2069 2070 2071
2088	469.9186	1406.7341	1406.7419	-5.55	0	17	7.9	1	U	K.HSQPWQVLVASR.G 2087 2089 2090

Supplementary information (SI) figure 9. 30 nM concentration, no surfactant, PSA only digest 2

Mascot Search Results

```
User                  : mervyn lewis
Email                 : m.lewis@bath.ac.uk
Search title          :
MS data file          : 150210sample13.mgf
Database              : SwissProt 2015_02 (547599 sequences; 195014757 residues)
Timestamp             : 11 Feb 2015 at 09:47:11 GMT
Enzyme                : Trypsin
Fixed modifications   : Carbamidomethyl (C)
Variable modifications : Oxidation (M)
Mass values           : Monoisotopic
Protein Mass          : Unrestricted
Peptide Mass Tolerance : ± 20 ppm
Fragment Mass Tolerance: ± 0.1 Da
Max Missed Cleavages  : 2
Instrument type       : ESI-QUAD-TOF
Number of queries     : 2068
Protein hits          : ALBU_BOVIN  Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4
                        ALBU_SHEEP  Serum albumin OS=Ovis aries GN=ALB PE=1 SV=1
                        ALBU_FELCA  Serum albumin OS=Felis catus GN=ALB PE=1 SV=1
                        ALBU_HUMAN  Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2
                        K2C1_HUMAN  Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6
                        ALBU_CANFA  Serum albumin OS=Canis familiaris GN=ALB PE=1 SV=3
                        KLK3_HUMAN  Prostate-specific antigen OS=Homo sapiens GN=KLK3 PE=1 SV=2
                        TRYP_PIG    Trypsin OS=Sus scrofa PE=1 SV=1
                        ALBU_RABIT  Serum albumin OS=Oryctolagus cuniculus GN=ALB PE=1 SV=2
                        ALBU_MOUSE  Serum albumin OS=Mus musculus GN=Alb PE=1 SV=3
                        MNTP_STRMK  Putative manganese efflux pump MntP OS=Stenotrophomonas maltophilia (strain K279a) GN=mntP PE=3 SV=1
                        NPC2_HUMAN  Epididymal secretory protein E1 OS=Homo sapiens GN=NPC2 PE=1 SV=1
                        K1C10_CANFA Keratin, type I cytoskeletal 10 OS=Canis familiaris GN=KRT10 PE=2 SV=1
                        KATG_METRJ  Catalase-peroxidase OS=Methylobacterium radiotolerans (strain ATCC 27329 / DSM 1819 / JCM 2831 / NBRC 15690 / NC
```

Select Summary Report

Format As [Select Summary (protein hits)] Help

Significance threshold p< 0.05 Max. number of hits AUTO

Standard scoring ○ MudPIT scoring ◉ Ions score or expect cut-off 0 Show sub-sets 0

Show pop-ups ◉ Suppress pop-ups ○ Require bold red ☐

Preferred taxonomy [All entries]

Re-Search ◉ All queries ○ Unassigned ○ Below homology threshold ○ Below identity threshold

1. ALBU_BOVIN **Mass:** 71244 **Score:** 606 **Matches:** 155(23) **Sequences:** 41(13) **emPAI:** 1.95
Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
828	333.1918	664.3690	664.3697	-0.98	1	13	9.9	1		K.KFWGK.Y 827 829 830 831
970	379.7155	757.4165	757.4156	1.09	0	48	0.0057	1		K.GACLLPK.I 969 971 972 973 974
1049	395.2389	788.4633	788.4644	-1.38	0	24	1.4	1		K.LVTDLTK.V 1044 1045 1046 1047 1048
1081	409.7163	817.4181	817.4181	-0.05	0	28	0.84	1		K.ATEEQLK.T 1078 1079 1080 1082
1128	424.2537	846.4929	846.4963	-4.05	1	31	0.24	1		R.LSQKFPK.A 1131 1132
1158	443.7114	885.4082	885.4080	0.23	0	30	0.13	1		K.DDSPDLPK.L 1157 1159 1160
1205	449.7439	897.4733	897.4742	-1.02	0	25	0.58	1		R.LCVLHEK.T 1206 1207 1208
1250	461.7486	921.4826	921.4807	1.99	0	46	0.0097	1	U	K.AEFVEVTK.L 1248 1249 1251 1252
1266	464.2503	926.4860	926.4861	-0.10	0	23	1.1	1		K.YLYEIAR.R 1262 1263 1264 1265
1291	487.7289	973.4432	973.4505	-7.49	0	25	0.33	1	U	K.DLGEEHFK.G 1292
1324	501.7941	1001.5736	1001.5757	-2.10	0	34	0.15	1	U	K.LVVSTQTALA.- 1321 1322 1323
1330	507.8118	1013.6091	1013.6121	-2.97	0	49	0.0042	1		K.QTALVELLK.H 1327 1328 1331
1637	534.7242	1067.4338	1067.4342	-0.39	0	23	0.17	1	U	K.QNCDQFEK.L 1635 1636 1638 1639 1640
1646	536.7562	1071.4979	1071.5019	-3.71	0	18	1.9	1	U	K.SHCIAEVEK.D 1645
1649	**358.1761**	**1071.5066**	**1071.5019**	**4.42**	**0**	**(1)**	**1.1e+002**	**2**	**U**	**K.SHCIAEVEK.D** 1647
1668	554.2605	1106.5064	1106.5066	-0.25	0	36	0.047	1		K.EACFAVEGPK.L 1669 1670
1682	569.7522	1137.4899	1137.4907	-0.68	0	38	0.012	1		K.CCTESLVNR.R 1683 1684 1685
1687	381.5759	1141.7059	1141.7070	-1.00	1	(14)	4.6	1		K.KQTALVELLK.H
1690	571.8612	1141.7078	1141.7070	0.70	1	33	0.063	1		K.KQTALVELLK.H 1691 1693
1714	582.3192	1162.6239	1162.6234	0.44	0	50	0.0042	1	U	K.LVNELTEFAK.T 1711 1712 1713 1715
1772	417.2093	1248.6061	1248.6139	-6.24	1	22	2.2	1	U	R.FKDLGEEHFK.G 1771
1779	**625.3144**	**1248.6142**	**1248.6139**	**0.29**	**1**	**(14)**	**15**	**3**	**U**	**R.FKDLGEEHFK.G**
1800	431.2066	1290.5979	1290.5948	2.40	0	23	0.86	1	U	K.ECCDKPLLEK.S 1799
1810	653.3612	1304.7079	1304.7088	-0.74	0	66	9.4e-005	1		K.HLVDEPQNLIK.Q 1807 1808 1809
1861	710.3493	1418.6841	1418.6864	-1.61	0	28	0.43	1		K.SLHTLFGDELCK.V 1862 1863
1866	473.9031	1418.6874	1418.6864	0.70	0	(27)	0.55	1		K.SLHTLFGDELCK.V 1860 1865 1867
1870	480.6080	1438.8020	1438.8045	-1.68	1	39	0.031	1		R.RHPEYAVSVLLR.L 1871 1872 1873
1877	722.3243	1442.6341	1442.6347	-0.45	0	45	0.0025	1	U	K.YICDNQDTISSK.L 1874 1875 1876
1886	732.2979	1462.5812	1462.5817	-0.32	0	(21)	0.12	1	U	K.TCVADESHAGCEK.S 1885 1888
1890	488.5345	1462.5818	1462.5817	0.09	0	31	0.011	1	U	K.TCVADESHAGCEK.S 1889 1891 1892
1902	740.4027	1478.7908	1478.7881	1.80	0	34	0.13	1	U	K.LGEYGFQNALIVR.Y 1901
1918	751.8102	1501.6058	1501.6065	-0.45	0	48	0.00033	1	U	K.EYEATLEECCAK.D 1917 1919 1920
1933	511.5988	1531.7746	1531.7738	0.53	1	13	24	1	U	K.LKECCDKPLLEK.S 1934
1951	518.8882	1553.6427	1553.6457	-1.93	0	22	0.19	1	U	K.DDPHACYSTVFDK.L 1949 1950 1952
1955	777.8291	1553.6436	1553.6457	-1.36	0	(13)	1.6	1	U	K.DDPHACYSTVFDK.L 1953 1954
1960	526.2622	1575.7648	1575.7603	2.88	0	20	3.2	1	U	K.LKPDPNTLCDEFK.A 1959 1961 1962
1981	547.3157	1638.9253	1638.9305	-3.19	1	43	0.011	1		R.KVPQVSTPTLVEVSR.S 1980 1982 1983 1984
2000	874.3557	1746.6969	1746.6978	-0.49	0	39	0.0018	1		K.YNGVFQECCQAEDK.G 1998 1999
2002	583.8918	1748.6535	1748.6553	-0.99	0	16	0.1	1		K.ECCHGDLLECADDR.A 2001
2009	599.2813	1794.8221	1794.8247	-1.44	1	3	85	1	U	K.DDPHACYSTVFDKLK.H
2012	605.6123	1813.8151	1813.8226	-4.13	1	18	2.2	1	U	R.LAKEYEATLEECCAK.D 2011
2019	627.6456	1879.9149	1879.9138	0.56	0	19	4.7	1	U	R.RPCFSALTPDETYVPK.A 2016 2017 2018
2030	643.2702	1926.7887	1926.7910	-1.21	1	16	0.51	1	U	K.CCAADDKEACFAVEGPK.L 2029 2031
2041	673.9926	2018.9560	2018.9619	-2.91	1	12	15	1	U	K.LKPDPNTLCDEFKADEK.K 2042
2044	529.2280	2112.8828	2112.8775	2.47	1	9	4.1	1		K.VHKECCHGDLLECADDR.A
2049	749.9820	2246.9242	2246.9354	-4.98	1	(2)	15	1		K.ECCHGDLLECADDRADLAK.Y
2051	562.7451	2246.9512	2246.9354	7.01	1	12	2.4	1		K.ECCHGDLLECADDRADLAK.Y
2055	831.4308	2491.2707	2491.2570	5.52	0	4	1.4e+002	1	U	K.GLVLIAFSQYLQQCPFDEHVK.L

7. KLK3_HUMAN **Mass:** 29293 **Score:** 60 **Matches:** 18(1) **Sequences:** 5(1) **emPAI:** 0.38

Prostate-specific antigen OS=Homo sapiens GN=KLK3 PE=1 SV=2

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
968	379.2488	756.4831	756.4858	-3.58	0	17	7.7	1	U	K.SVILLGR.H 965 966 967
1658	539.2560	1076.4974	1076.4961	1.27	0	21	1.5	1	U	R.IVGGWECEK.H 1659 1660 1661
1701	578.8217	1155.6288	1155.6288	0.02	1	46	0.0085	1	U	K.WIKDTIVANP.- 1702
1790	636.8375	1271.6604	1271.6609	-0.34	0	39	0.053	1	U	R.LSEPAELTDAVK.V 1787 1788 1789 1791 1792
1859	469.9204	1406.7394	1406.7419	-1.76	0	3	2.2e+002	1	U	K.HSQPWQVLVASR.G 1858

Supplementary information (SI) figure 10. 30 nM concentration, combined BSA & PSA digest 1

Mascot Search Results

```
User                  : mervyn lewis
Email                 : m.lewis@bath.ac.uk
Search title          :
MS data file          : 150210sample15.mgf
Database              : SwissProt 2015_02 (547599 sequences; 195014757 residues)
Timestamp             : 11 Feb 2015 at 09:08:56 GMT
Enzyme                : Trypsin
Fixed modifications   : Carbamidomethyl (C)
Variable modifications : Oxidation (M)
Mass values           : Monoisotopic
Protein Mass          : Unrestricted
Peptide Mass Tolerance : ± 20 ppm
Fragment Mass Tolerance: ± 0.1 Da
Max Missed Cleavages  : 2
Instrument type       : ESI-QUAD-TOF
Number of queries     : 1871
Protein hits          : ALBU_BOVIN Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4
                        ALBU_SHEEP Serum albumin OS=Ovis aries GN=ALB PE=1 SV=1
                        ALBU_FELCA Serum albumin OS=Felis catus GN=ALB PE=1 SV=1
                        KLK3_HUMAN Prostate-specific antigen OS=Homo sapiens GN=KLK3 PE=1 SV=2
                        ALBU_HUMAN Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2
                        TRYP_PIG   Trypsin OS=Sus scrofa PE=1 SV=1
                        ALBU_CANFA Serum albumin OS=Canis familiaris GN=ALB PE=1 SV=3
                        NPC2_HUMAN Epididymal secretory protein E1 OS=Homo sapiens GN=NPC2 PE=1 SV=1
                        ALBU_MOUSE Serum albumin OS=Mus musculus GN=Alb PE=1 SV=3
                        ALBU_RABIT Serum albumin OS=Oryctolagus cuniculus GN=ALB PE=1 SV=2
                        ALBU_EQUAS Serum albumin OS=Equus asinus GN=ALB PE=1 SV=1
```

Select Summary Report

Format As [Select Summary (protein hits)] Help

Significance threshold p< 0.05 Max. number of hits AUTO

Standard scoring ○ MudPIT scoring ◉ Ions score or expect cut-off 0 Show sub-sets 0

Show pop-ups ◉ Suppress pop-ups ○ Require bold red ☐

Preferred taxonomy [All entries]

Re-Search ◉ All queries ○ Unassigned ○ Below homology threshold ○ Below identity threshold

1. ALBU_BOVIN **Mass:** 71244 **Score:** 360 **Matches:** 128(12) **Sequences:** 38(7) **emPAI:** 1.46

Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
866	333.1922	664.3699	664.3697	0.34	1	10	18	1		K.KFWGK.Y 864 865 870
899	345.1902	688.3658	688.3656	0.25	0	7	85	5		K.AWSVAR.L
988	379.7144	757.4143	757.4156	-1.80	0	31	0.23	1		K.GACLLPK.I 983 984 985 986 987
1025	395.2379	788.4613	788.4644	-3.88	0	28	0.51	1		K.LVTDLTK.V 1022 1023 1024
1050	409.7166	817.4186	817.4181	0.54	0	22	3.7	1		K.ATEEQLK.T 1048 1052
1096	424.2553	846.4960	846.4963	-0.34	1	25	0.85	1		R.LSQKFPK.A 1094 1095
1113	443.7117	885.4089	885.4080	1.01	0	23	0.59	1		K.DDSPDLPK.L 1114 1115 1116
1177	449.7446	897.4747	897.4742	0.58	0	13	9.9	1		R.LCVLHEK.T 1174 1175 1176
1178	300.1657	897.4754	897.4742	1.29	0	(3)	1.1e+002	3		R.LCVLHEK.T
1220	461.7484	921.4822	921.4807	1.61	0	25	0.99	1	U	K.AEFVEVTK.L 1219 1221 1222
1227	464.2505	926.4865	926.4861	0.37	0	27	0.41	1		K.YLYEIAR.R 1224 1225 1226 1228 1229
1246	487.7322	973.4498	973.4505	-0.75	0	25	0.42	1	U	K.DLGEEHFK.G
1269	501.7947	1001.5748	1001.5757	-0.94	0	21	2.9	1	U	K.LVVSTQTALA.- 1270 1271 1272
1278	507.8130	1013.6114	1013.6121	-0.64	0	34	0.14	1		K.QTALVELLK.H 1276 1277 1279
1593	534.7251	1067.4357	1067.4342	1.44	0	37	0.0073	1	U	K.QNCDQFEK.L 1588 1589 1590 1591 1592
1595	536.7575	1071.5005	1071.5019	-1.32	0	23	0.66	1	U	K.SHCIAEVEK.D 1596
1615	554.2576	1106.5006	1106.5066	-5.47	0	20	1.5	1		K.EACFAVEGPK.L 1614
1629	569.7536	1137.4926	1137.4907	1.71	0	31	0.068	1		K.CCTESLVNR.R 1628 1630 1631
1635	571.8600	1141.7055	1141.7070	-1.36	1	29	0.14	1		K.KQTALVELLK.H 1632 1633 1634
1636	381.5772	1141.7098	1141.7070	2.42	1	(14)	4.8	1		K.KQTALVELLK.H
1656	582.3189	1162.6233	1162.6234	-0.04	0	43	0.024	1	U	K.LVNELTEFAK.T 1653 1654 1655
1693	417.2107	1248.6103	1248.6139	-2.90	1	17	6.3	1	U	R.FKDLGEEHFK.G
1709	431.2043	1290.5911	1290.5948	-2.82	0	11	12	1	U	K.ECCDKPLLEK.S 1711
1716	653.3646	1304.7146	1304.7088	4.45	0	40	0.039	1		K.HLVDEPQNLIK.Q 1713 1714 1715
1743	710.3503	1418.6860	1418.6864	-0.28	0	18	4.2	1		K.SLHTLFGDELCK.V 1742
1745	473.9036	1418.6890	1418.6864	1.87	0	(15)	9	1		K.SLHTLFGDELCK.V 1744

1752	480.6087	1438.8042	1438.8045	-0.19	1	16	6.1	1		R.RHPEYAVSVLLR.L 1245 1753
1756	722.3229	1442.6313	1442.6347	-2.42	0	49	0.001	1	U	K.YICDNQDTISSK.L 1754 1755 1757
1764	732.2962	1462.5779	1462.5817	-2.61	0	48	0.00023	1	U	K.TCVADESHAGCEK.S 1763
1768	488.5343	1462.5811	1462.5817	-0.38	0	(19)	0.19	1	U	K.TCVADESHAGCEK.S 1767 1769 1770
1775	740.3976	1478.7807	1478.7881	-5.07	0	24	1.5	1	U	K.LGEYGFQNALIVR.Y 1776 1777
1788	751.8095	1501.6044	1501.6065	-1.35	0	47	0.00033	1	U	K.EYEATLEECCAK.D 1785 1786 1787
1795	511.5996	1531.7768	1531.7738	1.98	1	12	25	1	U	K.LKECCDKPLLEK.S 1792
1810	777.8318	1553.6490	1553.6457	2.12	0	3	15	1	U	K.DDPHACYSTVFDK.L
1815	526.2631	1575.7674	1575.7603	4.49	0	9	41	1	U	K.LKPDPNTLCDEFK.A 1813 1814
1826	820.4703	1638.9261	1638.9305	-2.66	1	(10)	22	1		R.KVPQVSTPTLVEVSR.S
1827	547.3162	1638.9268	1638.9305	-2.24	1	32	0.14	1		R.KVPQVSTPTLVEVSR.S 1828 1830 1831 1832
1836	874.3589	1746.7033	1746.6978	3.19	0	20	0.17	1		K.YNGVFQECCQAEDK.G
1837	583.8904	1748.6494	1748.6553	-3.35	0	24	0.014	1		K.ECCHGDLLECADDR.A 1838
1840	599.2773	1794.8101	1794.8247	-8.12	1	3	66	1	U	K.DDPHACYSTVFDKLK.H
1845	627.6486	1879.9240	1879.9138	5.40	0	15	13	1	U	R.RPCFSALTPDETYVPK.A 1844 1846 1847
1854	643.2716	1926.7930	1926.7910	1.02	1	22	0.14	1	U	K.CCAADDKEACFAVEGPK.L 1852 1853 1855
1867	562.7411	2246.9355	2246.9354	0.02	1	6	6.2	1		K.ECCHGDLLECADDRADLAK.Y

2. ALBU_SHEEP **Mass:** 71139 **Score:** 105 **Matches:** 55(2) **Sequences:** 16(2) **emPAI:** 0.43

Serum albumin OS=Ovis aries GN=ALB PE=1 SV=1

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
866	333.1922	664.3699	664.3697	0.34	1	10	18	1		K.KFWGK.Y 864 865 870
899	345.1902	688.3658	688.3656	0.25	0	7	85	5		K.AWSVAR.L
988	379.7144	757.4143	757.4156	-1.80	0	31	0.23	1		K.GACLLPK.I 983 984 985 986 987
1025	395.2379	788.4613	788.4644	-3.88	0	28	0.51	1		K.IVTDLTK.V 1022 1023 1024
1096	424.2553	846.4960	846.4963	-0.34	1	25	0.85	1		R.LSQKFPK.A 1094 1095
1113	443.7117	885.4089	885.4080	1.01	0	23	0.59	1		K.DDSPDLPK.L 1114 1115 1116
1177	449.7446	897.4747	897.4742	0.58	0	13	9.9	1		R.LCVLHEK.T 1174 1175 1176
1178	300.1657	897.4754	897.4742	1.29	0	(3)	1.1e+002	3		R.LCVLHEK.T
1278	507.8130	1013.6114	1013.6121	-0.64	0	34	0.14	1		K.QTALVELLK.H 1276 1277 1279
1629	569.7536	1137.4926	1137.4907	1.71	0	31	0.068	1		K.CCTESLVNR.R 1628 1630 1631
1635	571.8600	1141.7055	1141.7070	-1.36	1	29	0.14	1		K.KQTALVELLK.H 1632 1633 1634
1636	381.5772	1141.7098	1141.7070	2.42	1	(14)	4.8	1		K.KQTALVELLK.H
1716	653.3646	1304.7146	1304.7088	4.45	0	40	0.039	1		K.HLVDEPQNLIK.K 1713 1714 1715
1743	710.3503	1418.6860	1418.6864	-0.28	0	18	4.2	1		K.SLHTLFGDELCK.V 1742
1745	473.9036	1418.6890	1418.6864	1.87	0	(15)	9	1		K.SLHTLFGDELCK.V 1744
1752	480.6087	1438.8042	1438.8045	-0.19	1	16	6.1	1		R.RHPEYAVSVLLR.L 1245 1753
1836	874.3589	1746.7033	1746.6978	3.19	0	20	0.17	1		K.YNGVFQECCQAEDK.G
1837	583.8904	1748.6494	1748.6553	-3.35	0	24	0.014	1		K.ECCHGDLLECADDR.A 1838
1867	562.7411	2246.9355	2246.9354	0.02	1	6	6.2	1		K.ECCHGDLLECADDRADLAK.Y

3. ALBU_FELCA **Mass:** 70611 **Score:** 88 **Matches:** 34(1) **Sequences:** 10(1) **emPAI:** 0.20

Serum albumin OS=Felis catus GN=ALB PE=1 SV=1

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
899	345.1902	688.3658	688.3656	0.25	0	7	85	5		K.AWSVAR.L
1050	409.7166	817.4186	817.4181	0.54	0	22	3.7	1		K.ATEEQLK.T 1048 1052
1096	424.2553	846.4960	846.4963	-0.34	1	25	0.85	1		R.LSQKFPK.A 1094 1095
1177	449.7446	897.4747	897.4742	0.58	0	13	9.9	1		R.LCVLHEK.T 1174 1175 1176
1178	300.1657	897.4754	897.4742	1.29	0	(3)	1.1e+002	3		R.LCVLHEK.T
1227	464.2505	926.4865	926.4861	0.37	0	27	0.41	1		K.YLYEIAR.R 1224 1225 1226 1228 1229
1629	569.7536	1137.4926	1137.4907	1.71	0	31	0.068	1		K.CCTESLVNR.R 1628 1630 1631
1775	740.3976	1478.7807	1478.7881	-5.07	0	24	1.5	1		K.LGEYGFQNALLVR.Y 1776 1777
1826	820.4703	1638.9261	1638.9305	-2.66	1	(10)	22	1		K.KVPQVSTPTLVEVSR.S
1827	547.3162	1638.9268	1638.9305	-2.24	1	32	0.14	1		K.KVPQVSTPTLVEVSR.S 1828 1830 1831 1832
1837	583.8904	1748.6494	1748.6553	-3.35	0	24	0.014	1		K.ECCHGDLLECADDR.A 1838
1867	562.7411	2246.9355	2246.9354	0.02	1	6	6.2	1		K.ECCHGDLLECADDRADLAK.Y

4. KLK3_HUMAN **Mass:** 29293 **Score:** 86 **Matches:** 14(3) **Sequences:** 4(1) **emPAI:** 0.24

Prostate-specific antigen OS=Homo sapiens GN=KLK3 PE=1 SV=2

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
982	379.2496	756.4847	756.4858	-1.35	0	23	1.5	1	U	K.SVILLGR.H 979 980 981
1606	539.2569	1076.4993	1076.4961	2.97	0	21	1.6	1	U	R.IVGGWECEK.H 1607
1649	578.8213	1155.6280	1155.6288	-0.67	1	7	72	1	U	K.WIKDTIVANP.- 1648
1701	636.8359	1271.6573	1271.6609	-2.79	0	55	0.0012	1	U	R.LSEPAELTDAVK.V 1702 1703 1704 1705 1706

Supplementary information (SI) figure 11. 30 nM concentration, combined BSA & PSA digest 2

Mascot Search Results

```
User                  : mervyn lewis
Email                 : m.lewis@bath.ac.uk
Search title          :
MS data file          : 150630sample15.mgf
Database              : SwissProt 2015_07 (548872 sequences; 195617763 residues)
Timestamp             : 2 Jul 2015 at 09:32:52 GMT
Enzyme                : Trypsin
Fixed modifications   : Carbamidomethyl (C)
Variable modifications : Oxidation (M)
Mass values           : Monoisotopic
Protein Mass          : Unrestricted
Peptide Mass Tolerance : ± 20 ppm
Fragment Mass Tolerance: ± 0.1 Da
Max Missed Cleavages  : 2
Instrument type       : ESI-QUAD-TOF
Number of queries     : 1967
Protein hits          : ALBU_BOVIN Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4
                        ALBU_FELCA Serum albumin OS=Felis catus GN=ALB PE=1 SV=1
                        ALBU_SHEEP Serum albumin OS=Ovis aries GN=ALB PE=1 SV=1
                        ALBU_HUMAN Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2
                        ALBU_CANFA Serum albumin OS=Canis familiaris GN=ALB PE=1 SV=3
                        ALBU_MESAU Serum albumin OS=Mesocricetus auratus GN=ALB PE=1 SV=1
                        ALBU_MOUSE Serum albumin OS=Mus musculus GN=Alb PE=1 SV=3
                        CRP_HUMAN  C-reactive protein OS=Homo sapiens GN=CRP PE=1 SV=1
                        CRP_MOUSE  C-reactive protein OS=Mus musculus OX=10090 GN=Crp PE=1 SV=2
                        ALBU_EQUAS Serum albumin OS=Equus asinus OX=9793 GN=ALB PE=1 SV=1
                        FETA_PIG   Alpha-fetoprotein OS=Sus scrofa OX=9823 GN=AFP PE=2 SV=1
                        RSMH_PELUB Ribosomal RNA small subunit methyltransferase H OS=Pelagibacter ubique (strain HTCC1062) GN=rsmH PE=3 SV=1
```

Select Summary Report

Format As [Select Summary (protein hits)] Help

Significance threshold p< 0.05 Max. number of hits AUTO

Standard scoring ○ MudPIT scoring ◉ Ions score or expect cut-off 0 Show sub-sets 0

Show pop-ups ◉ Suppress pop-ups ○ Require bold red ☐

Preferred taxonomy [All entries]

Re-Search ◉ All queries ○ Unassigned ○ Below homology threshold ○ Below identity threshold

1. ALBU_BOVIN **Mass:** 71244 **Score:** 1528 **Matches:** 247(47) **Sequences:** 39(15) **emPAI:** 2.69
Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
910	333.1939	664.3733	664.3697	5.44	1	19	2.3	1		K.KFWGK.Y 911 912
934	345.1897	688.3649	688.3656	-1.08	0	13	23	2		K.AWSVAR.L
964	379.7155	757.4165	757.4156	1.16	0	41	0.031	1		K.GACLLPK.I 963 965 966 967 968 969
981	395.2378	788.4611	788.4644	-4.11	0	32	0.23	1		K.LVTDLTK.V 980 982 986 987 988 990 991 992
1009	424.2546	846.4946	846.4963	-2.00	1	9	35	1		R.LSQKFPK.A 1011
1102	449.7398	897.4650	897.4742	-10.26	0	13	9.2	1		R.LCVLHEK.T 1103
1104	300.1669	897.4788	897.4742	5.11	0	(1)	1.8e+002	1		R.LCVLHEK.T
1121	461.7471	921.4796	921.4807	-1.19	0	38	0.052	1	U	K.AEFVEVTK.L 1119 1120 1122 1123 1124 1125 1126 1127
1144	464.2494	926.4843	926.4861	-1.95	0	29	0.26	1		K.YLYEIAR.R 1134 1135 1136 1137 1138 1139 1140 1141 1142 1143 1145 11
1183	501.7948	1001.5750	1001.5757	-0.75	0	27	0.89	1	U	K.LVVSTQTALA.- 1174 1175 1176 1177 1178 1179 1180 1182 1184 1185 118
1194	507.8130	1013.6115	1013.6121	-0.60	0	39	0.039	1		K.QTALVELLK.H 1193 1195 1196 1197 1198 1199
1361	534.7264	1067.4382	1067.4342	3.75	0	16	1.1	1	U	K.QNCDQFEK.L 1360
1404	569.7500	1137.4855	1137.4907	-4.54	0	38	0.0084	1		K.CCTESLVNR.R 1405
1407	571.8591	1141.7036	1141.7070	-3.04	1	29	0.23	1		K.KQTALVELLK.H 1408 1409 1410 1411
1425	582.3183	1162.6221	1162.6234	-1.05	0	61	0.00028	1	U	K.LVNELTEFAK.T 1417 1418 1419 1420 1421 1422 1423 1424 1426
1456	417.2123	1248.6152	1248.6139	1.03	1	11	32	1	U	R.FKDLGEEHFK.G 1454 1455 1457 1458 1459
1491	653.3601	1304.7056	1304.7088	-2.48	0	57	0.0008	1		K.HLVDEPQNLIK.Q 1484 1485 1486 1487 1488 1489 1490 1492 1493 1494 14
1517	710.3485	1418.6824	1418.6864	-2.83	0	37	0.055	1		K.SLHTLFGDELCK.V 1516 1518
1524	473.9022	1418.6847	1418.6864	-1.20	0	(32)	0.18	1		K.SLHTLFGDELCK.V 1519 1520 1521 1522 1523 1525 1526
1536	480.6083	1438.8032	1438.8045	-0.91	1	50	0.0025	1		R.RHPEYAVSVLLR.L 1535 1537 1538 1539 1540 1541 1542 1543 1544 1545 1
1552	722.3248	1442.6351	1442.6347	0.27	0	44	0.0034	1	U	K.YICDNQDTISSK.L 1549 1550 1551 1553 1554
1560	488.5341	1462.5804	1462.5817	-0.86	0	17	0.27	1	U	K.TCVADESHAGCEK.S 1561 1562 1563
1584	740.4014	1478.7883	1478.7881	0.09	0	82	2.2e-006	1		K.LGEYGFQNALIVR.Y 1582 1583 1585 1586 1587
1598	751.8115	1501.6085	1501.6065	1.38	0	32	0.014	1	U	K.EYEATLEECCAK.D 1597 1599
1610	511.5974	1531.7705	1531.7738	-2.19	1	5	1.4e+002	4	U	K.LKECCDKPLLEK.S 1607 1608
1209	518.8899	1553.6479	1553.6457	1.44	0	15	1	1	U	K.DDPHACYSTVFDK.L 1622
1634	784.3737	1566.7328	1566.7354	-1.66	0	74	8.9e-006	1	U	K.DAFLGSFLYEYSR.R 1630 1631 1632 1633 1635 1636 1637 1638
1643	526.2605	1575.7597	1575.7603	-0.39	0	31	0.27	1	U	K.LKPDPNTLCDEFK.A 1640 1641 1642 1644 1645 1646 1647
1659	547.3162	1638.9268	1638.9305	-2.23	1	74	8.6e-006	1		R.KVPQVSTPTLVEVSR.S 1660 1661 1662 1663 1664 1665 1666 1667 1668 166
1709	575.6147	1723.8222	1723.8273	-2.94	0	(19)	3.7	1		R.MPCTEDYLSLILNR.L 1708
1710	862.9189	1723.8233	1723.8273	-2.33	0	42	0.017	1		R.MPCTEDYLSLILNR.L 1711
1737	599.2801	1794.8186	1794.8247	-3.41	1	4	56	1	U	K.DDPHACYSTVFDKLK.H 1739
1768	627.6450	1879.9133	1879.9138	-0.30	0	21	3	1	U	R.RPCFSALTPDETYVPK.A 1767 1769 1770
1771	630.3141	1887.9206	1887.9195	0.58	0	26	0.8	1		R.HPYFYAPELLYYANK.Y 1772 1773
1780	636.6451	1906.9136	1906.9135	0.06	0	37	0.06	1	U	K.LFTFHADICTLPDTEK.Q 1775 1776 1777 1778 1779 1781
1188	505.7484	2018.9645	2018.9619	1.32	1	(2)	3.1e+002	1	U	K.LKPDPNTLCDEFKADEK.K 1810
1814	673.9959	2018.9658	2018.9619	1.93	1	6	78	2	U	K.LKPDPNTLCDEFKADEK.K
1831	682.3471	2044.0194	2044.0206	-0.60	1	45	0.01	1		R.RHPYFYAPELLYYANK.Y 1825 1826 1827 1828 1829 1830 1832 1833 1834 18
1384	562.7446	2246.9495	2246.9354	6.25	1	16	3.2	1		K.ECCHGDLLECADDRADLAK.Y 1871
1909	820.0634	2457.1682	2457.1733	-2.08	1	4	1e+002	1	U	K.DAIPENLPPLTADFAEDKDVCK.N
1924	829.7081	2486.1026	2486.1028	-0.10	1	12	5.1	1		K.YNGVFQECCQAEDKGACLLPK.I 1920 1921 1922 1923
1933	831.4255	2491.2547	2491.2570	-0.90	0	39	0.051	1	U	K.GLVLIAFSQYLQQCPFDEHVK.L 1929 1930 1931 1932 1934
1961	703.1423	3510.6753	3510.6647	3.04	2	(19)	3	1	U	K.SHCIAEVEKDAIPENLPPLTADFAEDKDVCK.N 1960 1962 1963 1964 1965
1967	878.6797	3510.6895	3510.6647	7.08	2	33	0.1	1	U	K.SHCIAEVEKDAIPENLPPLTADFAEDKDVCK.N 1957 1958 1959 1966

8. CRP_HUMAN **Mass:** 25194 **Score:** 167 **Matches:** 47(3) **Sequences:** 7(1) **emPAI:** 1.39
C-reactive protein OS=Homo sapiens GN=CRP PE=1 SV=1

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
941	354.7085	707.4024	707.4007	2.47	0	29	0.29	1		K.AFVFPK.E 940 942 943 944 945 946 947
1008	418.7547	835.4948	835.4956	-1.00	1	34	0.12	1		R.KAFVFPK.E 1000 1001 1002 1003 1004 1005 1006 1007
1386	564.7727	1127.5308	1127.5346	-3.36	0	44	0.0065	1	U	K.ESDTSYVSLK.A 1387 1388 1389
1401	568.7845	1135.5545	1135.5550	-0.39	0	23	1.5	1	U	R.GYSIFSYATK.R 1395 1396 1397 1398 1399 1400
1621	516.9440	1547.8101	1547.8096	0.31	1	27	0.87	1	U	K.RQDNEILIFWSK.D 1618 1619 1620
1746	910.9664	1819.9182	1819.9145	2.04	0	24	1.8	1	U	K.YEVQGEVFTKPQLWP.- 1740 1742 1743 1744 1745
1855	711.7165	2132.1276	2132.1306	-1.43	1	27	0.82	1	U	R.ALKYEVQGEVFTKPQLWP.- 1849 1850 1851 1852 1853 1854 1856 1858

Supplementary information (SI) figure 12. 500 nM concentration, combined BSA & CRP digest 0.1 µL injection volume

Mascot Search Results

```
User                  : mervyn lewis
Email                 : m.lewis@bath.ac.uk
Search title          :
MS data file          : 150702sample16.mgf
Database              : SwissProt 2015_07 (548872 sequences; 195617763 residues)
Timestamp             : 7 Jul 2015 at 07:59:25 GMT
Enzyme                : Trypsin
Fixed modifications   : Carbamidomethyl (C)
Variable modifications : Oxidation (M)
Mass values           : Monoisotopic
Protein Mass          : Unrestricted
Peptide Mass Tolerance : ± 20 ppm
Fragment Mass Tolerance: ± 0.1 Da
Max Missed Cleavages  : 2
Instrument type       : ESI-QUAD-TOF
Number of queries     : 3278
Protein hits          : ALBU_BOVIN Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4
                        ALBU_SHEEP Serum albumin OS=Ovis aries GN=ALB PE=1 SV=1
                        ALBU_FELCA Serum albumin OS=Felis catus GN=ALB PE=1 SV=1
                        ALBU_CANFA Serum albumin OS=Canis familiaris GN=ALB PE=1 SV=3
                        CRP_HUMAN  C-reactive protein OS=Homo sapiens GN=CRP PE=1 SV=1
                        ALBU_MOUSE Serum albumin OS=Mus musculus GN=Alb PE=1 SV=3
                        ALBU_MESAU Serum albumin OS=Mesocricetus auratus GN=ALB PE=1 SV=1
                        ALBU_MACFA Serum albumin OS=Macaca fascicularis GN=ALB PE=2 SV=1
                        ALBU_HUMAN Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2
                        CRP_MOUSE  C-reactive protein OS=Mus musculus OX=10090 GN=Crp PE=1 SV=2
                        RSMH_PELUB Ribosomal RNA small subunit methyltransferase H OS=Pelagibacter ubique (strain HTCC1062) GN=rsmH PE=3 SV=1
                        ALBU_EQUAS Serum albumin OS=Equus asinus GN=ALB PE=1 SV=1
                        ALBU_RABIT Serum albumin OS=Oryctolagus cuniculus GN=ALB PE=1 SV=2
                        ALBU_HORSE Serum albumin OS=Equus caballus GN=ALB PE=1 SV=1
                        FETA_PIG   Alpha-fetoprotein OS=Sus scrofa OX=9823 GN=AFP PE=2 SV=1
                        SCP_CHIOP  Sarcoplasmic calcium-binding protein (Fragment) OS=Chionoecetes opilio PE=1 SV=1
                        RFCL_METS3 Replication factor C large subunit OS=Methanobrevibacter smithii (strain PS / ATCC 35061 / DSM 861) GN=rfcL PE=3
```

Select Summary Report

Format As [Select Summary (protein hits)] Help

Significance threshold p< 0.05 Max. number of hits AUTO

Standard scoring / MudPIT scoring / Ions score or expect cut-off 0 Show sub-sets 0

Show pop-ups / Suppress pop-ups / Require bold red

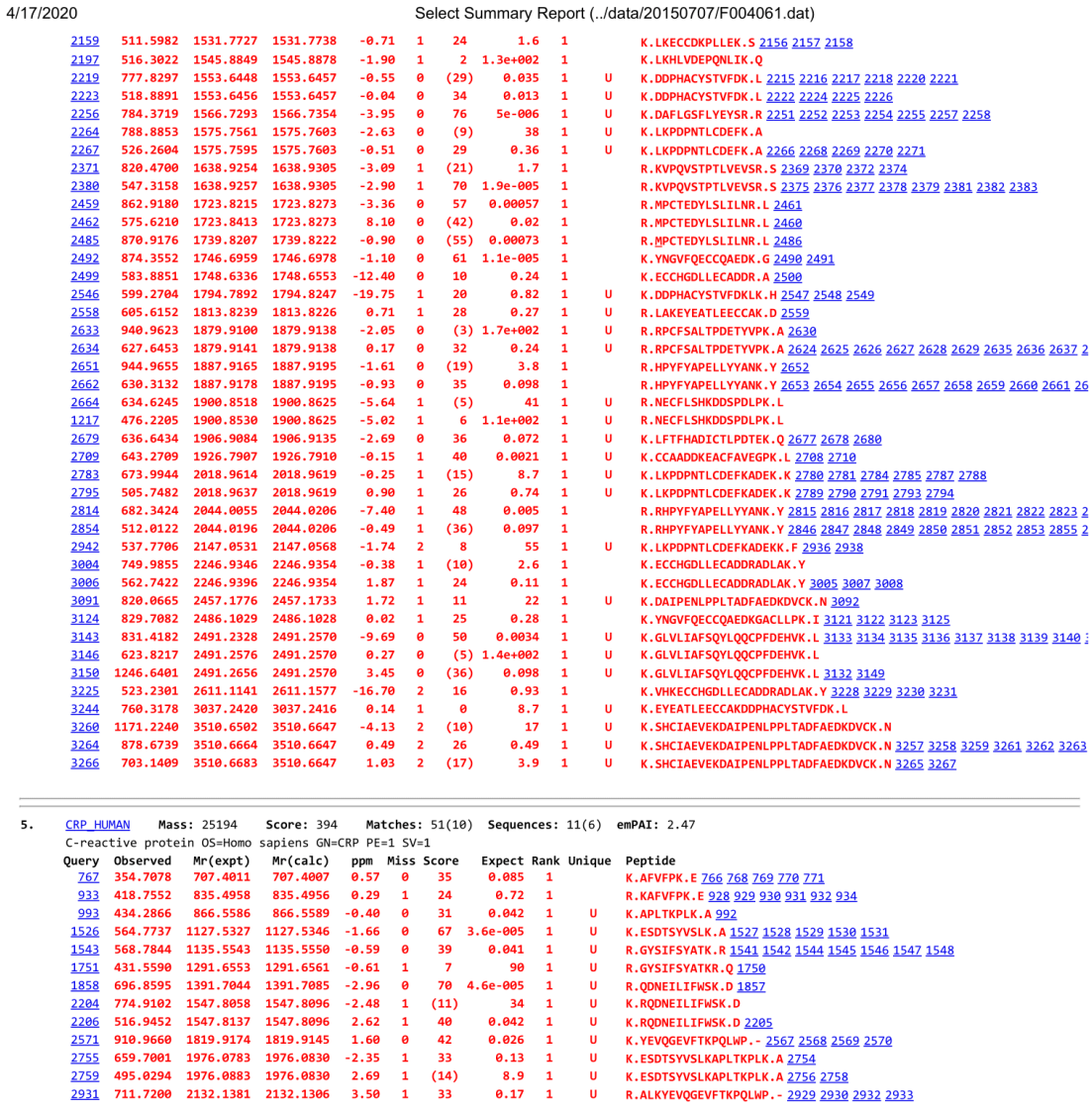
Preferred taxonomy [All entries]

Re-Search / All queries / Unassigned / Below homology threshold / Below identity threshold

1. ALBU_BOVIN **Mass:** 71244 **Score:** 3265 **Matches:** 385(93) **Sequences:** 56(24) **emPAI:** 10.91

Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
685	333.1898	664.3651	664.3697	-6.92	1	26	0.82	2		K.KFWGK.Y 675 678 681 682 683 684 687 688 689 690 692 693 695 697
722	345.1898	688.3650	688.3656	-0.89	0	17	9.2	5		K.AWSVAR.L 721 723
835	379.7152	757.4159	757.4156	0.35	0	29	0.47	1		K.GACLLPK.I 833 834 836 837 838 839
873	395.2394	788.4643	788.4644	-0.05	0	43	0.017	1		K.LVTDLTK.V 868 869 870 871 872
899	409.7179	817.4212	817.4181	3.74	0	31	0.47	1		K.ATEEQLK.T 896 897 898 900
972	424.2561	846.4977	846.4963	1.62	1	21	2.2	1		R.LSQKFPK.A 970 971 973 974 975
1013	443.7120	885.4095	885.4080	1.75	0	50	0.0013	1		K.DDSPDLPK.L 1009 1010 1011 1012
1123	449.7445	897.4745	897.4742	0.32	0	36	0.056	1		R.LCVLHEK.T 1119 1120 1121 1122
1125	300.1672	897.4797	897.4742	6.14	0	(12)	14	2		R.LCVLHEK.T 1117 1118 1124
1179	461.7474	921.4802	921.4807	-0.59	0	44	0.014	1	U	K.AEFVEVTK.L 1178 1180 1181 1182 1183
1191	464.2495	926.4845	926.4861	-1.79	0	35	0.059	1		K.YLYEIAR.R 1190 1192 1193 1194 1195 1196 1197 1198
1269	487.7326	973.4507	973.4505	0.23	0	10	14	1		K.DLGEEHFK.G
1344	501.7948	1001.5751	1001.5757	-0.61	0	30	0.38	1	U	K.LVVSTQTALA.- 1342 1343 1345 1346
1358	507.8112	1013.6079	1013.6121	-4.16	0	63	0.00014	1		K.QTALVELLK.H 1356 1357
1439	534.7238	1067.4331	1067.4342	-1.07	0	38	0.005	1	U	K.QNCDQFEK.L 1436 1437 1438 1440 1441
1449	358.1701	1071.4884	1071.5019	-12.54	0	(19)	1.5	1	U	K.SHCIAEVEK.D 1450 1453
1451	536.7581	1071.5016	1071.5019	-0.24	0	22	0.89	1	U	K.SHCIAEVEK.D 1452
1555	569.7508	1137.4870	1137.4907	-3.20	0	37	0.013	1		K.CCTESLVNR.R 1554 1556 1557
1562	571.8610	1141.7074	1141.7070	0.36	1	57	0.00025	1		K.KQTALVELLK.H 1560 1561 1563 1564
1565	381.5793	1141.7159	1141.7070	7.80	1	(28)	0.2	1		K.KQTALVELLK.H 1559
1597	582.3183	1162.6220	1162.6234	-1.18	0	60	0.00036	1	U	K.LVNELTEFAK.T 1596 1598 1599 1600 1601 1602
1714	625.3151	1248.6156	1248.6139	1.36	1	(20)	4	2		R.FKDLGEEHFK.G 1712 1713
1717	417.2126	1248.6159	1248.6139	1.65	1	29	0.42	1		R.FKDLGEEHFK.G 1715 1716 1718 1719
1741	642.3603	1282.7060	1282.7034	2.09	0	26	0.76	1		R.HPEYAVSVLLR.L 1742
1746	431.2049	1290.5928	1290.5948	-1.53	0	17	2.8	1		K.ECCDKPLLEK.S 1745
1771	653.3596	1304.7047	1304.7088	-3.18	0	59	0.00055	1		K.HLVDEPQNLIK.Q 1769 1770 1772 1773 1774 1775
1863	700.3481	1398.6816	1398.6853	-2.67	0	60	0.00024	1		K.TVMENFVAFVDK.C 1864 1865
1878	708.3479	1414.6812	1414.6803	0.69	0	(44)	0.0094	1		K.TVMENFVAFVDK.C 1877
1888	710.3511	1418.6876	1418.6864	0.84	0	61	0.00025	1		K.SLHTLFGDELCK.V 1889 1890 1891
1895	473.9036	1418.6890	1418.6864	1.85	0	(45)	0.0084	1		K.SLHTLFGDELCK.V 1892 1893 1894 1896
1925	480.6038	1438.7895	1438.8045	-10.39	1	63	0.00013	1		R.RHPEYAVSVLLR.L 1915 1916 1917 1918 1919 1920 1921 1922 1923 1924 ;
1948	722.3246	1442.6346	1442.6347	-0.08	0	58	0.00013	1	U	K.YICDNQDTISSK.L 1944 1945 1946 1947 1949
1981	488.5340	1462.5800	1462.5817	-1.13	0	39	0.0019	1	U	K.TCVADESHAGCEK.S 1982 1983 1984
1985	732.2977	1462.5808	1462.5817	-0.57	0	(27)	0.026	1	U	K.TCVADESHAGCEK.S 1986
2036	740.4011	1478.7877	1478.7881	-0.33	0	112	2.5e-009	1		K.LGEYGFQNALIVR.Y 2035 2037 2038 2039 2040
2108	751.8105	1501.6065	1501.6065	0.01	0	58	3.4e-005	1	U	K.EYEATLEECCAK.D 2109 2110 2111
2112	756.4247	1510.8348	1510.8355	-0.47	0	28	0.38	1		K.VPQVSTPTLVEVSR.S



4/17/2020 Select Summary Report (../data/20150707/F004061.dat)

2159	511.5982	1531.7727	1531.7738	-0.71	1	24	1.6	1		K.LKECCDKPLLEK.S 2156 2157 2158
2197	516.3022	1545.8849	1545.8878	-1.90	1	2	1.3e+002	1		K.LKHLVDEPQNLIK.Q
2219	777.8297	1553.6448	1553.6457	-0.55	0	(29)	0.035	1	U	K.DDPHACYSTVFDK.L 2215 2216 2217 2218 2220 2221
2223	518.8891	1553.6456	1553.6457	-0.04	0	34	0.013	1	U	K.DDPHACYSTVFDK.L 2222 2224 2225 2226
2256	784.3719	1566.7293	1566.7354	-3.95	0	76	5e-006	1	U	K.DAFLGSFLYEYSR.R 2251 2252 2253 2254 2255 2257 2258
2264	788.8853	1575.7561	1575.7603	-2.63	0	(9)	38	1	U	K.LKPDPNTLCDEFK.A
2267	526.2604	1575.7595	1575.7603	-0.51	0	29	0.36	1	U	K.LKPDPNTLCDEFK.A 2266 2268 2269 2270 2271
2371	820.4700	1638.9254	1638.9305	-3.09	1	(21)	1.7	1		R.KVPQVSTPTLVEVSR.S 2369 2370 2372 2374
2380	547.3158	1638.9257	1638.9305	-2.90	1	70	1.9e-005	1		R.KVPQVSTPTLVEVSR.S 2375 2376 2377 2378 2379 2381 2382 2383
2459	862.9180	1723.8215	1723.8273	-3.36	0	57	0.00057	1		R.MPCTEDYLSLILNR.L 2461
2462	575.6210	1723.8413	1723.8273	8.10	0	(42)	0.02	1		R.MPCTEDYLSLILNR.L 2460
2485	870.9176	1739.8207	1739.8222	-0.90	0	(55)	0.00073	1		R.MPCTEDYLSLILNR.L 2486
2492	874.3552	1746.6959	1746.6978	-1.10	0	61	1.1e-005	1		K.YNGVFQECCQAEDK.G 2490 2491
2499	583.8851	1748.6336	1748.6553	-12.40	0	10	0.24	1		K.ECCHGDLLECADDR.A 2500
2546	599.2704	1794.7892	1794.8247	-19.75	1	20	0.82	1	U	K.DDPHACYSTVFDKLK.H 2547 2548 2549
2558	605.6152	1813.8239	1813.8226	0.71	1	28	0.27	1	U	R.LAKEYEATLEECCAK.D 2559
2633	940.9623	1879.9100	1879.9138	-2.05	0	(3)	1.7e+002	1	U	R.RPCFSALTPDETYVPK.A 2630
2634	627.6453	1879.9141	1879.9138	0.17	0	32	0.24	1	U	R.RPCFSALTPDETYVPK.A 2624 2625 2626 2627 2628 2629 2635 2636 2637 2
2651	944.9655	1887.9165	1887.9195	-1.61	0	(19)	3.8	1		R.HPYFYAPELLYYANK.Y 2652
2662	630.3132	1887.9178	1887.9195	-0.93	0	35	0.098	1		R.HPYFYAPELLYYANK.Y 2653 2654 2655 2656 2657 2658 2659 2660 2661 26
2664	634.6245	1900.8518	1900.8625	-5.64	1	(5)	41	1	U	R.NECFLSHKDDSPDLPK.L
1217	476.2205	1900.8530	1900.8625	-5.02	1	6	1.1e+002	1	U	R.NECFLSHKDDSPDLPK.L
2679	636.6434	1906.9084	1906.9135	-2.69	0	36	0.072	1	U	K.LFTFHADICTLPDTEK.Q 2677 2678 2680
2709	643.2709	1926.7907	1926.7910	-0.15	1	40	0.0021	1	U	K.CCAADDKEACFAVEGPK.L 2708 2710
2783	673.9944	2018.9614	2018.9619	-0.25	1	(15)	8.7	1	U	K.LKPDPNTLCDEFKADEK.K 2780 2781 2784 2785 2787 2788
2795	505.7482	2018.9637	2018.9619	0.90	1	26	0.74	1	U	K.LKPDPNTLCDEFKADEK.K 2789 2790 2791 2793 2794
2814	682.3424	2044.0055	2044.0206	-7.40	1	48	0.005	1		R.RHPYFYAPELLYYANK.Y 2815 2816 2817 2818 2819 2820 2821 2822 2823 2
2854	512.0122	2044.0196	2044.0206	-0.49	1	(36)	0.097	1		R.RHPYFYAPELLYYANK.Y 2846 2847 2848 2849 2850 2851 2852 2853 2855 2
2942	537.7706	2147.0531	2147.0568	-1.74	2	8	55	1	U	K.LKPDPNTLCDEFKADEKK.F 2936 2938
3004	749.9855	2246.9346	2246.9354	-0.38	1	(10)	2.6	1		K.ECCHGDLLECADDRADLAK.Y
3006	562.7422	2246.9396	2246.9354	1.87	1	24	0.11	1		K.ECCHGDLLECADDRADLAK.Y 3005 3007 3008
3091	820.0665	2457.1776	2457.1733	1.72	1	11	22	1	U	K.DAIPENLPPLTADFAEDKDVCK.N 3092
3124	829.7082	2486.1029	2486.1028	0.02	1	25	0.28	1		K.YNGVFQECCQAEDKGACLLPK.I 3121 3122 3123 3125
3143	831.4182	2491.2328	2491.2570	-9.69	0	50	0.0034	1	U	K.GLVLIAFSQYLQQCPFDEHVK.L 3133 3134 3135 3136 3137 3138 3139 3140 3
3146	623.8217	2491.2576	2491.2570	0.27	0	(5)	1.4e+002	1	U	K.GLVLIAFSQYLQQCPFDEHVK.L
3150	1246.6401	2491.2656	2491.2570	3.45	0	(36)	0.098	1	U	K.GLVLIAFSQYLQQCPFDEHVK.L 3132 3149
3225	523.2301	2611.1141	2611.1577	-16.70	2	16	0.93	1		K.VHKECCHGDLLECADDRADLAK.Y 3228 3229 3230 3231
3244	760.3178	3037.2420	3037.2416	0.14	1	0	8.7	1	U	K.EYEATLEECCAKDDPHACYSTVFDK.L
3260	1171.2240	3510.6502	3510.6647	-4.13	2	(10)	17	1	U	K.SHCIAEVEKDAIPENLPPLTADFAEDKDVCK.N
3264	878.6739	3510.6664	3510.6647	0.49	2	26	0.49	1	U	K.SHCIAEVEKDAIPENLPPLTADFAEDKDVCK.N 3257 3258 3259 3261 3262 3263
3266	703.1409	3510.6683	3510.6647	1.03	2	(17)	3.9	1	U	K.SHCIAEVEKDAIPENLPPLTADFAEDKDVCK.N 3265 3267

5. CRP_HUMAN **Mass:** 25194 **Score:** 394 **Matches:** 51(10) **Sequences:** 11(6) **emPAI:** 2.47

C-reactive protein OS=Homo sapiens GN=CRP PE=1 SV=1

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
767	354.7078	707.4011	707.4007	0.57	0	35	0.085	1		K.AFVFPK.E 766 768 769 770 771
933	418.7552	835.4958	835.4956	0.29	1	24	0.72	1		R.KAFVFPK.E 928 929 930 931 932 934
993	434.2866	866.5586	866.5589	-0.40	0	31	0.042	1	U	K.APLTKPLK.A 992
1526	564.7737	1127.5327	1127.5346	-1.66	0	67	3.6e-005	1	U	K.ESDTSYVSLK.A 1527 1528 1529 1530 1531
1543	568.7844	1135.5543	1135.5550	-0.59	0	39	0.041	1	U	R.GYSIFSYATK.R 1541 1542 1544 1545 1546 1547 1548
1751	431.5590	1291.6553	1291.6561	-0.61	1	7	90	1	U	R.GYSIFSYATKR.Q 1750
1858	696.8595	1391.7044	1391.7085	-2.96	0	70	4.6e-005	1	U	R.QDNEILIFWSK.D 1857
2204	774.9102	1547.8058	1547.8096	-2.48	1	(11)	34	1	U	K.RQDNEILIFWSK.D
2206	516.9452	1547.8137	1547.8096	2.62	1	40	0.042	1	U	K.RQDNEILIFWSK.D 2205
2571	910.9660	1819.9174	1819.9145	1.60	0	42	0.026	1	U	K.YEVQGEVFTKPQLWP.- 2567 2568 2569 2570
2755	659.7001	1976.0783	1976.0830	-2.35	1	33	0.13	1	U	K.ESDTSYVSLKAPLTKPLK.A 2754
2759	495.0294	1976.0883	1976.0830	2.69	1	(14)	8.9	1	U	K.ESDTSYVSLKAPLTKPLK.A 2756 2758
2931	711.7200	2132.1381	2132.1306	3.50	1	33	0.17	1	U	R.ALKYEVQGEVFTKPQLWP.- 2929 2930 2932 2933

Supplementary information (SI) figure 13. 500 nM concentration, combined BSA & CRP digest 0.3 µL injection volume

Mascot Search Results

```
User                  : mervyn lewis
Email                 : m.lewis@bath.ac.uk
Search title          :
MS data file          : 150630sample17.mgf
Database              : SwissProt 2015_07 (548872 sequences; 195617763 residues)
Timestamp             : 2 Jul 2015 at 08:04:10 GMT
Enzyme                : Trypsin
Fixed modifications   : Carbamidomethyl (C)
Variable modifications : Oxidation (M)
Mass values           : Monoisotopic
Protein Mass          : Unrestricted
Peptide Mass Tolerance : ± 20 ppm
Fragment Mass Tolerance: ± 0.1 Da
Max Missed Cleavages  : 2
Instrument type       : ESI-QUAD-TOF
Number of queries     : 1908
Protein hits          : ALBU_BOVIN Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4
                        ALBU_SHEEP Serum albumin OS=Ovis aries GN=ALB PE=1 SV=1
                        ALBU_FELCA Serum albumin OS=Felis catus GN=ALB PE=1 SV=1
                        ALBU_CANFA Serum albumin OS=Canis familiaris GN=ALB PE=1 SV=3
                        IL6_HUMAN  Interleukin-6 OS=Homo sapiens GN=IL6 PE=1 SV=1
                        ALBU_HUMAN Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2
                        ALBU_MESAU Serum albumin OS=Mesocricetus auratus GN=ALB PE=1 SV=1
                        ALBU_MOUSE Serum albumin OS=Mus musculus GN=Alb PE=1 SV=3
                        ATPA_SALAI ATP synthase subunit alpha OS=Salinispora arenicola (strain CNS-205) OX=391037 GN=atpA PE=3 SV=1
                        FETA_PIG   Alpha-fetoprotein OS=Sus scrofa OX=9823 GN=AFP PE=2 SV=1
                        TATA_BART1 Sec-independent protein translocase protein TatA OS=Bartonella tribocorum (strain CIP 105476 / IBS 506) GN=tatA P
```

Select Summary Report

Format As: Select Summary (protein hits) — Help

Significance threshold p< 0.05 — Max. number of hits AUTO

Standard scoring / MudPIT scoring / Ions score or expect cut-off 0 — Show sub-sets 0

Show pop-ups / Suppress pop-ups — Require bold red

Preferred taxonomy: All entries

Re-Search — All queries / Unassigned / Below homology threshold / Below identity threshold

1. ALBU_BOVIN **Mass:** 71244 **Score:** 1474 **Matches:** 249(43) **Sequences:** 38(13) **emPAI:** 3.23
Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
945	333.1924	664.3702	664.3697	0.72	1	25	0.62	1		K.KFWGK.Y 943 944 946 948 949
966	345.1907	688.3668	688.3656	1.72	0	19	6.2	1		K.AWSVAR.L
983	379.7160	757.4175	757.4156	2.48	0	26	0.88	1		K.GACLLPK.I 984
995	395.2395	788.4644	788.4644	0.01	0	26	0.9	1		K.LVTDLTK.V 991 992 993 994 996 997 998 1000 1001 1002
1004	424.2550	846.4954	846.4963	-1.10	1	28	0.5	1		R.LSQKFPK.A 1005 1006 1007
1056	449.7436	897.4727	897.4742	-1.71	0	12	13	1		R.LCVLHEK.T 1057 1058 1059
1063	300.1668	897.4787	897.4742	4.99	0	(7)	42	1		R.LCVLHEK.T
1075	461.7471	921.4797	921.4807	-1.17	0	33	0.18	1	U	K.AEFVEVTK.L 1074 1076 1077 1078 1079 1080
1095	464.2499	926.4853	926.4861	-0.93	0	30	0.21	1		K.YLYEIAR.R 1086 1087 1088 1089 1090 1091 1092 1093 1094 1096 1097 10
1130	501.7946	1001.5747	1001.5757	-1.00	0	27	0.8	1	U	K.LVVSTQTALA.- 1131 1132 1133 1134 1135 1136
1141	507.8128	1013.6111	1013.6121	-1.01	0	51	0.0028	1		K.QTALVELLK.H 1139 1140 1142 1143 1144 1145 1146
1344	534.7228	1067.4310	1067.4342	-3.00	0	29	0.037	1	U	K.QNCDQFEK.L 1343
1361	569.7550	1137.4954	1137.4907	4.15	0	34	0.041	1		K.CCTESLVNR.R 1359 1360
1369	571.8599	1141.7052	1141.7070	-1.57	1	48	0.0021	1		K.KQTALVELLK.H 1364 1365 1366 1367 1368
1382	582.3178	1162.6210	1162.6234	-2.07	0	49	0.0052	1	U	K.LVNELTEFAK.T 1379 1380 1381 1383 1384
1402	417.2127	1248.6161	1248.6139	1.80	1	13	19	1	U	R.FKDLGEEHFK.G 1403 1404 1405 1406 1407 1408
1422	653.3616	1304.7087	1304.7088	-0.09	0	61	0.00031	1		K.HLVDEPQNLIK.Q 1418 1419 1420 1421 1423 1424 1425 1426 1427
1457	710.3493	1418.6840	1418.6864	-1.71	0	(24)	1.2	1		K.SLHTLFGDELCK.V 1458 1459
1464	473.9033	1418.6882	1418.6864	1.28	0	42	0.019	1		K.SLHTLFGDELCK.V 1461 1462 1463 1465 1466 1467 1468 1469
1479	480.6089	1438.8050	1438.8045	0.36	1	61	0.00023	1		R.RHPEYAVSVLLR.L 1472 1473 1474 1475 1476 1477 1478 1480 1481 1482 1
1494	722.3240	1442.6334	1442.6347	-0.97	0	38	0.012	1	U	K.YICDNQDTISSK.L 1488 1489 1490 1491 1492 1493
1503	488.5344	1462.5813	1462.5817	-0.26	0	31	0.013	1	U	K.TCVADESHAGCEK.S 1499 1500 1501 1502 1504
1522	740.4008	1478.7871	1478.7881	-0.68	0	86	1e-006	1		K.LGEYGFQNALIVR.Y 1516 1517 1518 1519 1520 1521
1540	751.8134	1501.6122	1501.6065	3.85	0	25	0.072	1	U	K.EYEATLEECCAK.D 1541 1542
1554	511.5972	1531.7699	1531.7738	-2.58	1	13	19	1	U	K.LKECCDKPLLEK.S 1553
1569	518.8878	1553.6415	1553.6457	-2.67	0	15	0.9	1	U	K.DDPHACYSTVFDK.L 1566 1567 1568
1580	784.3739	1566.7333	1566.7354	-1.36	0	69	2.6e-005	1	U	K.DAFLGSFLYEYSR.R 1575 1576 1577 1578 1579 1581 1582 1583
1586	526.2614	1575.7623	1575.7603	1.26	0	28	0.49	1	U	K.LKPDPNTLCDEFK.A 1587 1588 1589
1607	547.3168	1638.9286	1638.9305	-1.14	1	58	0.00031	1		R.KVPQVSTPTLVEVSR.S 1606 1608 1609 1610 1611 1612 1613 1614 1615 161
1636	575.6171	1723.8296	1723.8273	1.31	0	26	0.76	1		R.MPCTEDYLSLILNR.L
1637	580.9457	1739.8154	1739.8222	-3.92	0	(6)	54	1		R.MPCTEDYLSLILNR.L
1658	599.2833	1794.8281	1794.8247	1.92	1	12	11	1	U	K.DDPHACYSTVFDKLK.H 1656 1657
1669	627.6466	1879.9181	1879.9138	2.28	0	29	0.41	1	U	K.RPCFSALTPDETYVPK.A 1666 1668 1670 1671 1672 1673 1674 1675
1677	630.3104	1887.9093	1887.9195	-5.44	0	26	0.75	1		R.HPYFYAPELLYYANK.Y
1684	636.6450	1906.9131	1906.9135	-0.21	0	37	0.063	1	U	K.LFTFHADICTLPDTEK.Q 1683 1685
1709	505.7483	2018.9641	2018.9619	1.08	1	(12)	16	1	U	K.LKPDPNTLCDEFKADEK.K 1710
1712	673.9955	2018.9647	2018.9619	1.37	1	21	2.2	1	U	K.LKPDPNTLCDEFKADEK.K 1716
1721	682.3396	2043.9968	2044.0206	-11.64	1	31	0.26	1		R.RHPYFYAPELLYYANK.Y 1718 1719 1720 1722 1723 1724 1725 1726 1727 17
1747	512.0118	2044.0183	2044.0206	-1.16	1	(12)	24	1		R.RHPYFYAPELLYYANK.Y 1746
1772	562.7416	2246.9372	2246.9354	0.79	1	1	19	1		K.ECCHGDLLECADDRADLAK.Y 1770
1834	829.7037	2486.0892	2486.1028	-5.51	1	17	1.4	1		K.YNGVFQECCQAEDKGACLLPK.I 1831 1832 1833
1850	831.4285	2491.2636	2491.2570	2.65	0	28	0.58	1	U	K.GLVLIAFSQYLQQCPFDEHVK.L 1851
1896	703.1407	3510.6669	3510.6647	0.64	2	(20)	2	1	U	K.SHCIAEVEKDAIPENLPPLTADFAEDKDVCK.N 1893 1894 1895
1897	878.6748	3510.6701	3510.6647	1.55	2	23	0.97	1	U	K.SHCIAEVEKDAIPENLPPLTADFAEDKDVCK.N 1898 1899 1900 1901

5. IL6_HUMAN **Mass:** 23931 **Score:** 304 **Matches:** 48(12) **Sequences:** 10(5) **emPAI:** 2.24
Interleukin-6 OS=Homo sapiens GN=IL6 PE=1 SV=1

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1103	490.2651	978.5156	978.5134	2.19	0	34	0.15	1	U	K.EFLQSSLR.A 1104 1105 1106
1119	494.8122	987.6098	987.6117	-1.94	0	41	0.0085	1	U	K.VLIQFLQK.K 1112 1113 1114 1115 1116 1117 1118
1350	560.8195	1119.6245	1119.6288	-3.86	0	40	0.037	1	U	R.YILDGISALR.K 1347 1348 1349 1351
1392	416.9146	1247.7219	1247.7237	-1.44	1	23	1	1	U	R.YILDGISALRK.E 1393 1394
1395	624.8698	1247.7251	1247.7237	1.12	1	(11)	12	1	U	R.YILDGISALRK.E 1396
1436	663.3583	1324.7020	1324.6986	2.54	0	82	2.1e-006	1	U	K.EALAENNLNLPK.M 1433 1434 1435 1437 1438 1439
1444	671.3607	1340.7068	1340.7088	-1.50	1	(10)	28	1	U	R.SFKEFLQSSLR.A 1445
1448	447.9102	1340.7086	1340.7088	-0.15	1	22	2.3	1	U	R.SFKEFLQSSLR.A 1446 1447 1449 1450 1451
1679	945.9060	1889.7975	1889.7924	2.67	0	33	0.019	1	U	K.DGCFQSGFNEETCLVK.I 1678
1697	993.5229	1985.0313	1985.0317	-0.21	0	52	0.0028	1	U	K.NLDAITTPDPTTNASLLTK.L 1695 1696 1698 1699
1700	662.6869	1985.0388	1985.0317	3.60	0	(19)	4.5	1	U	K.NLDAITTPDPTTNASLLTK.L 1701
1763	737.0620	2208.1642	2208.1473	7.64	0	10	33	1	U	K.LQAQNQWLQDMTTHLILR.S
1764	738.3941	2212.1605	2212.1779	-7.89	0	23	1.8	1	U	K.IITGLLEFEVYLEYLQNR.F

Supplementary information (SI) figure 14. 500 nM concentration, combined BSA & IL-6 digest 0.1µL injection volume

Mascot Search Results

```
User                  : mervyn lewis
Email                 : m.lewis@bath.ac.uk
Search title          :
MS data file          : 150630sample19.mgf
Database              : SwissProt 2015_07 (548872 sequences; 195617763 residues)
Timestamp             : 2 Jul 2015 at 09:36:16 GMT
Enzyme                : Trypsin
Fixed modifications   : Carbamidomethyl (C)
Variable modifications : Oxidation (M)
Mass values           : Monoisotopic
Protein Mass          : Unrestricted
Peptide Mass Tolerance : ± 20 ppm
Fragment Mass Tolerance: ± 0.1 Da
Max Missed Cleavages  : 2
Instrument type       : ESI-QUAD-TOF
Number of queries     : 1719
Protein hits          : ALBU_BOVIN Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4
                        ALBU_FELCA Serum albumin OS=Felis catus GN=ALB PE=1 SV=1
                        ALBU_SHEEP Serum albumin OS=Ovis aries GN=ALB PE=1 SV=1
                        ALBU_CANFA Serum albumin OS=Canis familiaris GN=ALB PE=1 SV=3
                        ALBU_MOUSE Serum albumin OS=Mus musculus GN=Alb PE=1 SV=3
                        ALBU_HUMAN Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2
                        ALBU_MACFA Serum albumin OS=Macaca fascicularis GN=ALB PE=2 SV=1
                        IL8_CERAT  Interleukin-8 OS=Cercocebus atys GN=CXCL8 PE=3 SV=1
                        RSMH_PELUB Ribosomal RNA small subunit methyltransferase H OS=Pelagibacter ubique (strain HTCC1062) GN=rsmH PE=3 SV=1
                        IL8_HUMAN  Interleukin-8 OS=Homo sapiens GN=CXCL8 PE=1 SV=1
                        FETA_PIG   Alpha-fetoprotein OS=Sus scrofa OX=9823 GN=AFP PE=2 SV=1
                        ENRN_BPT3  Endodeoxyribonuclease 1 OS=Enterobacteria phage T3 GN=3 PE=4 SV=1
                        RPOC_GEOBB DNA-directed RNA polymerase subunit beta' OS=Geobacter bemidjiensis (strain Bem / ATCC BAA-1014 / DSM 16622) GN=r
                        RR3_CYACA  30S ribosomal protein S3, chloroplastic OS=Cyanidium caldarium GN=rps3 PE=3 SV=1
```

Select Summary Report

Format As: Select Summary (protein hits) — Help

Significance threshold p< 0.05 — Max. number of hits AUTO

Standard scoring / MudPIT scoring — Ions score or expect cut-off 0 — Show sub-sets 0

Show pop-ups / Suppress pop-ups — Require bold red

Preferred taxonomy: All entries

Re-Search — All queries / Unassigned / Below homology threshold / Below identity threshold

1. ALBU_BOVIN **Mass:** 71244 **Score:** 1350 **Matches:** 194(41) **Sequences:** 31(13) **emPAI:** 1.57
Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
968	333.1920	664.3694	664.3697	-0.45	1	12	12	1		K.KFWGK.Y
977	345.1906	688.3667	688.3656	1.46	0	10	49	6		K.AWSVAR.L 982
1006	379.7151	757.4156	757.4156	0.01	0	29	0.47	1		K.GACLLPK.I 1004 1005 1007 1008 1009
1022	395.2390	788.4634	788.4644	-1.26	0	23	1.7	1		K.LVTDLTK.V 1019 1020 1021
1038	424.2528	846.4910	846.4963	-6.29	1	9	39	1		R.LSQKFPK.A 1037
1086	449.7478	897.4811	897.4742	7.69	0	7	38	2		R.LCVLHEK.T
1096	461.7474	921.4802	921.4807	-0.63	0	43	0.019	1	U	K.AEFVEVTK.L 1088 1089 1090 1091 1092 1093 1094 1095
1113	464.2502	926.4858	926.4861	-0.38	0	29	0.27	1		K.YLYEIAR.R 1100 1101 1102 1103 1104 1105 1106 1107 1108 1109 1111 111
1141	501.7954	1001.5763	1001.5757	0.55	0	38	0.057	1	U	K.LVVSTQTALA.- 1136 1137 1138 1139 1140 1142 1143 1144 1145 1146 1147
1156	507.8134	1013.6123	1013.6121	0.22	0	53	0.0016	1		K.QTALVELLK.H 1151 1152 1153 1154 1155
1358	569.7533	1137.4920	1137.4907	1.16	0	36	0.021	1		K.CCTESLVNR.R 1359 1360
1367	571.8608	1141.7071	1141.7070	0.07	1	33	0.061	1		K.KQTALVELLK.H 1363 1364 1365 1366 1368
1370	381.5772	1141.7097	1141.7070	2.37	1	(5)	41	2		K.KQTALVELLK.H
1375	582.3177	1162.6209	1162.6234	-2.13	0	51	0.0035	1	U	K.LVNELTEFAK.T 1376 1377 1378 1379 1380 1381 1382 1383
1396	417.2105	1248.6096	1248.6139	-3.45	1	3	1.5e+002	1		R.FKDLGEEHFK.G
1412	653.3597	1304.7049	1304.7088	-3.00	0	40	0.041	1		K.HLVDEPQNLIK.Q 1402 1403 1404 1405 1406 1407 1408 1409 1410 1411
1428	710.3507	1418.6868	1418.6864	0.28	0	(32)	0.17	1		K.SLHTLFGDELCK.V 1429
1430	473.9041	1418.6904	1418.6864	2.80	0	32	0.19	1		K.SLHTLFGDELCK.V 1431 1432 1433 1434
1439	480.6085	1438.8038	1438.8045	-0.49	1	45	0.0087	1		R.RHPEYAVSVLLR.L 1437 1438 1440 1441 1442 1443 1444 1445 1446 1447 14
1452	722.3261	1442.6375	1442.6347	1.94	0	36	0.022	1	U	K.YICDNQDTISSK.L 1451 1453 1454 1455 1456
1483	740.4005	1478.7864	1478.7881	-1.19	0	77	7.6e-006	1	U	K.LGEYGFQNALIVR.Y 1480 1481 1482 1484 1485 1486
1011	383.9524	1531.7807	1531.7738	4.49	1	11	31	1		K.LKECCDKPLLEK.S
1513	511.6013	1531.7822	1531.7738	5.49	1	(7)	95	1		K.LKECCDKPLLEK.S
1528	784.3749	1566.7353	1566.7354	-0.11	0	83	1.2e-006	1	U	K.DAFLGSFLYEYSR.R 1524 1525 1526 1527 1529 1530 1531 1532 1533
1539	526.2616	1575.7631	1575.7603	1.80	0	16	8.9	1	U	K.LKPDPNTLCDEFK.A 1535 1536 1537 1538 1540
1562	547.3165	1638.9278	1638.9305	-1.66	1	46	0.0048	1		R.KVPQVSTPTLVEVSR.S 1561 1563 1564 1565 1566 1567
1614	599.2828	1794.8266	1794.8247	1.04	1	7	40	1	U	K.DDPHACYSTVFDKLK.H
1632	627.6463	1879.9172	1879.9138	1.77	0	21	2.9	1	U	R.RPCFSALTPDETYVPK.A 1631 1633 1634 1635 1636
1642	636.6456	1906.9151	1906.9135	0.81	0	30	0.33	1	U	K.LFTFHADICTLPDTEK.Q 1639 1640 1641
1656	673.9984	2018.9735	2018.9619	5.76	1	8	53	1	U	K.LKPDPNTLCDEFKADEK.K
1666	682.3466	2044.0179	2044.0206	-1.33	1	26	0.81	1		R.RHPYFYAPELLYYANK.Y 1660 1661 1662 1663 1664 1665 1667 1668 1669 167
1679	829.7092	2486.1059	2486.1028	1.21	1	19	1.1	1		K.YNGVFQECCQAEDKGACLLPK.I 1680 1681 1682
1686	831.4269	2491.2588	2491.2570	0.72	0	47	0.0077	1	U	K.GLVLIAFSQYLQQCPFDEHVK.L 1687 1688 1689 1690 1691 1692 1693
1694	623.8265	2491.2768	2491.2570	7.96	0	(5)	1.3e+002	1	U	K.GLVLIAFSQYLQQCPFDEHVK.L
1716	878.6766	3510.6774	3510.6647	3.64	2	(14)	8.5	1	U	K.SHCIAEVEKDAIPENLPPLTADFAEDKDVCK.N 1712 1713 1714 1715
1718	703.1434	3510.6807	3510.6647	4.56	2	30	0.22	1	U	K.SHCIAEVEKDAIPENLPPLTADFAEDKDVCK.N 1717 1719

10. IL8_HUMAN **Mass:** 11376 **Score:** 31 **Matches:** 26(1) **Sequences:** 4(1) **emPAI:** 0.70
Interleukin-8 OS=Homo sapiens GN=CXCL8 PE=1 SV=1

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
1031	416.2089	830.4032	830.4035	-0.33	0	29	0.26	1	U	K.ENWVQR.V 1028 1029 1030 1032 1033
1045	437.7214	873.4282	873.4266	1.88	0	38	0.045	1		R.ELCLDPK.E 1039 1040 1041 1042 1043 1044 1046 1047 1048 1049 1050
1350	368.8661	1103.5763	1103.5764	-0.04	0	2	3e+002	1		K.TYSKPFHPK.F
1586	589.6398	1765.8975	1765.9033	-3.27	0	18	6.7	1	U	R.VIESGPHCANTEIIVK.L 1587 1588 1589 1590 1592 1594

Supplementary information (SI) figure 15. 500 nM concentration, combined BSA & IL-8 digest 0.1 µL injection volume

Mascot Search Results

```
User                  : mervyn lewis
Email                 : m.lewis@bath.ac.uk
Search title          :
MS data file          : 150702sample18.mgf
Database              : SwissProt 2015_07 (548872 sequences; 195617763 residues)
Timestamp             : 7 Jul 2015 at 08:03:56 GMT
Enzyme                : Trypsin
Fixed modifications   : Carbamidomethyl (C)
Variable modifications : Oxidation (M)
Mass values           : Monoisotopic
Protein Mass          : Unrestricted
Peptide Mass Tolerance : ± 20 ppm
Fragment Mass Tolerance: ± 0.1 Da
Max Missed Cleavages  : 2
Instrument type       : ESI-QUAD-TOF
Number of queries     : 2827
Protein hits          : ALBU_BOVIN Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4
                        ALBU_SHEEP Serum albumin OS=Ovis aries GN=ALB PE=1 SV=1
                        ALBU_FELCA Serum albumin OS=Felis catus GN=ALB PE=1 SV=1
                        ALBU_CANFA Serum albumin OS=Canis familiaris GN=ALB PE=1 SV=3
                        ALBU_MESAU Serum albumin OS=Mesocricetus auratus GN=ALB PE=1 SV=1
                        ALBU_MOUSE Serum albumin OS=Mus musculus GN=Alb PE=1 SV=3
                        ALBU_MACFA Serum albumin OS=Macaca fascicularis GN=ALB PE=2 SV=1
                        ALBU_HUMAN Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2
                        IL8_HUMAN  Interleukin-8 OS=Homo sapiens GN=CXCL8 PE=1 SV=1
                        SCP_CHIOP  Sarcoplasmic calcium-binding protein (Fragment) OS=Chionoecetes opilio PE=1 SV=1
                        RSMH_PELUB Ribosomal RNA small subunit methyltransferase H OS=Pelagibacter ubique (strain HTCC1062) GN=rsmH PE=3 SV=1
                        ALBU_HORSE Serum albumin OS=Equus caballus GN=ALB PE=1 SV=1
                        ALBU_RABIT Serum albumin OS=Oryctolagus cuniculus GN=ALB PE=1 SV=2
                        ALBU_EQUAS Serum albumin OS=Equus asinus OX=9793 GN=ALB PE=1 SV=1
                        GLYA_PEPD6 Serine hydroxymethyltransferase OS=Peptoclostridium difficile (strain 630) GN=glyA PE=3 SV=1
                        CYAS_METAC Cysteate synthase OS=Methanosarcina acetivorans (strain ATCC 35395 / DSM 2834 / JCM 12185 / C2A) GN=MA_3297 PE=1
```

Select Summary Report

Format As [Select Summary (protein hits)] Help

Significance threshold p< 0.05 Max. number of hits AUTO

Standard scoring ○ MudPIT scoring ◉ Ions score or expect cut-off 0 Show sub-sets 0

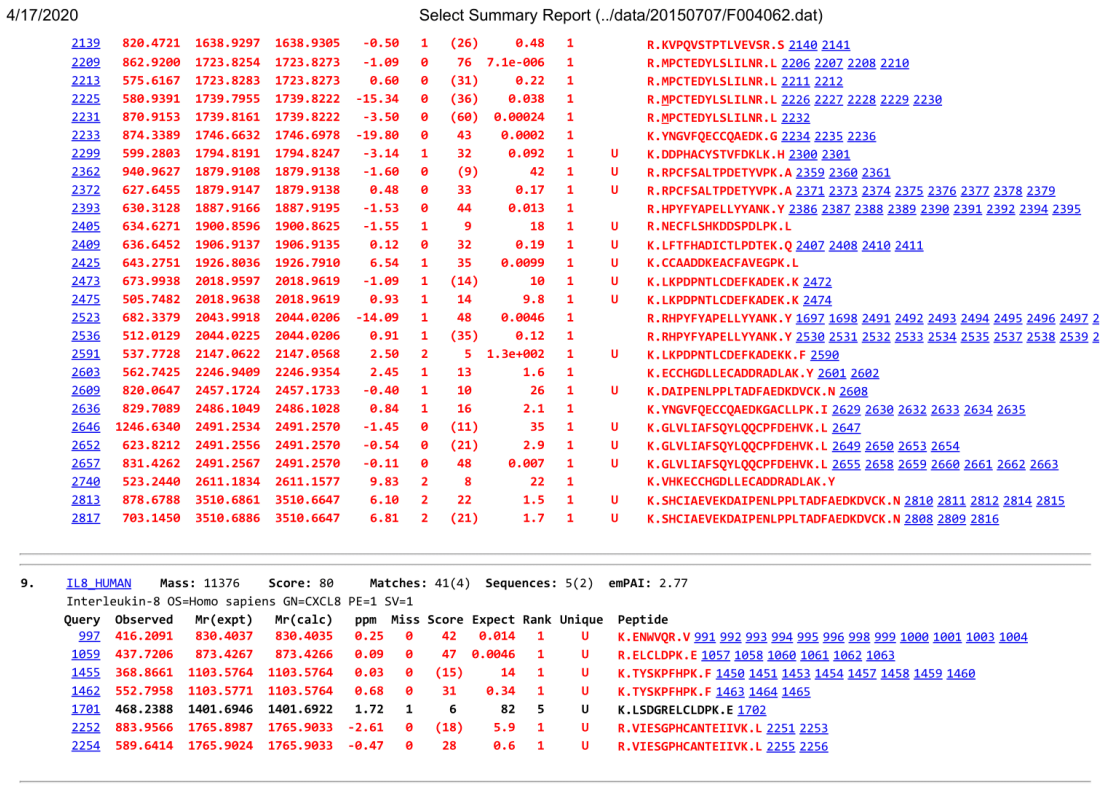
Show pop-ups ◉ Suppress pop-ups ○ Require bold red ☐

Preferred taxonomy [All entries]

Re-Search ◉ All queries ○ Unassigned ○ Below homology threshold ○ Below identity threshold

1. ALBU_BOVIN **Mass:** 71244 **Score:** 3194 **Matches:** 386(110) **Sequences:** 50(24) **emPAI:** 6.59
Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
810	333.1895	664.3645	664.3697	-7.77	1	26	0.76	2		K.KFWGK.Y 794 795 796 797 799 800 801 802 803 804 806 807 808 811 812
917	379.7147	757.4149	757.4156	-1.00	0	38	0.05	1		K.GACLLPK.I 916 918 919 920 921 922 923 924 925 926
953	395.2389	788.4633	788.4644	-1.42	0	41	0.029	1		K.LVTDLTK.V 945 946 947 948 949 950 951 952 954 956
1040	424.2556	846.4966	846.4963	0.28	1	27	0.51	1		R.LSQKFPK.A 1038 1039 1041 1043 1044 1045
1071	443.7115	885.4085	885.4080	0.64	0	27	0.27	1		K.DDSPDLPK.L
1141	300.1599	897.4579	897.4742	-18.22	0	(12)	13	2		R.LCVLHEK.T 1140 1152 1153 1156 1160
1149	449.7444	897.4743	897.4742	0.15	0	32	0.15	1		R.LCVLHEK.T 1144 1145 1146 1147 1148 1150
1202	461.7472	921.4798	921.4807	-1.05	0	46	0.0086	1	U	K.AEFVEVTK.L 1197 1198 1199 1200 1201 1203 1204
1215	464.2496	926.4847	926.4861	-1.57	0	31	0.17	1		K.YLYEIAR.R 1209 1210 1211 1212 1213 1214 1216 1217 1218
1316	501.7939	1001.5732	1001.5757	-2.49	0	36	0.096	1	U	K.LVVSTQTALA.- 1312 1313 1314 1315
1322	507.8118	1013.6091	1013.6121	-2.95	0	51	0.0023	1		K.QTALVELLK.H 1323 1324 1325 1326 1327
1420	534.7235	1067.4325	1067.4342	-1.59	0	37	0.0072	1	U	K.QNCDQFEK.L 1416 1417 1418 1419 1421
1437	358.1712	1071.4919	1071.5019	-9.34	0	15	4.4	1	U	K.SHCIAEVEK.D
1490	569.7525	1137.4904	1137.4907	-0.25	0	54	0.00034	1		K.CCTESLVNR.R 1481 1482 1483 1484 1485 1486 1487 1488 1489
1497	571.8600	1141.7054	1141.7070	-1.41	1	34	0.043	1		K.KQTALVELLK.H 1494 1495 1496 1498
1502	381.5786	1141.7140	1141.7070	6.13	1	(27)	0.22	1		K.KQTALVELLK.H 1500 1501 1503
1552	582.3177	1162.6209	1162.6234	-2.10	0	66	0.00012	1	U	K.LVNELTEFAK.T 1550 1551 1553 1554
1602	417.2041	1248.5905	1248.6139	-18.71	1	28	0.43	1		R.FKDLGEEHFK.G 1596 1598 1599 1601 1609 1610
1614	642.3577	1282.7008	1282.7034	-1.98	0	27	0.6	1		R.HPEYAVSVLLR.L 1615 1616
1617	431.2051	1290.5934	1290.5948	-1.07	0	4	68	3		K.ECCDKPLLEK.S
1631	653.3610	1304.7075	1304.7088	-1.01	0	46	0.011	1		K.HLVDEPQNLIK.Q 1629 1630 1632 1633 1634
1707	708.3459	1414.6773	1414.6803	-2.07	0	27	0.46	1		K.TVMENFVAFVDK.C 1706
1723	710.3496	1418.6846	1418.6864	-1.29	0	45	0.0093	1		K.SLHTLFGDELCK.V 1718 1719 1720 1721 1722
1726	473.9026	1418.6860	1418.6864	-0.30	0	(43)	0.014	1		K.SLHTLFGDELCK.V 1724 1725 1727 1728 1729
1747	480.6078	1438.8016	1438.8045	-1.98	1	65	7.7e-005	1		R.RHPEYAVSVLLR.L 1744 1745 1746 1748 1749 1750 1751
1765	722.3244	1442.6342	1442.6347	-0.41	0	60	9e-005	1	U	K.YICDNQDTISSK.L 1760 1761 1762 1763 1764 1766 1767 1768 1769 1770
1788	732.2946	1462.5746	1462.5817	-4.87	0	26	0.031	1	U	K.TCVADESHAGCEK.S
1789	488.5334	1462.5785	1462.5817	-2.18	0	(26)	0.037	1	U	K.TCVADESHAGCEK.S 1790 1791 1792
1839	740.4015	1478.7885	1478.7881	0.24	0	108	5.5e-009	1		K.LGEYGFQNALIVR.Y 1836 1837 1838 1840 1841 1842 1843 1844 1845 1846
1901	751.8099	1501.6052	1501.6065	-0.84	0	59	2.3e-005	1	U	K.EYEATLEECCAK.D 1898 1899 1900 1902
1904	756.4212	1510.8278	1510.8355	-5.12	0	25	0.94	1		K.VPQVSTPTLVEVSR.S 1903
1939	511.5977	1531.7713	1531.7738	-1.64	1	13	20	1		K.LKECCDKPLLEK.S 1936 1937 1938 1940
1976	516.3017	1545.8833	1545.8878	-2.97	1	12	12	1		K.LKHLVDEPQNLIK.Q 1977
1992	777.8267	1553.6389	1553.6457	-4.35	0	(17)	0.5	1	U	K.DDPHACYSTVFDK.L 1991 1993 1994 1999
1996	518.8884	1553.6432	1553.6457	-1.58	0	27	0.053	1	U	K.DDPHACYSTVFDK.L 1995 1997 1998
2014	784.3748	1566.7351	1566.7354	-0.20	0	80	2.4e-006	1	U	K.DAFLGSFLYEYSR.R 2011 2012 2013 2015 2016 2017 2018 2019
2024	526.2598	1575.7577	1575.7603	-1.64	0	32	0.2	1	U	K.LKPDPNTLCDEFK.A 2021 2022 2023 2025 2026 2027
2137	547.3164	1638.9275	1638.9305	-1.83	1	66	4.6e-005	1		R.KVPQVSTPTLVEVSR.S 2132 2133 2134 2135 2136



4/17/2020 Select Summary Report (../data/20150707/F004062.dat)

2139	820.4721	1638.9297	1638.9305	-0.50	1	(26)	0.48	1		R.KVPQVSTPTLVEVSR.S 2140 2141
2209	862.9200	1723.8254	1723.8273	-1.09	0	76	7.1e-006	1		R.MPCTEDYLSLILNR.L 2206 2207 2208 2210
2213	575.6167	1723.8283	1723.8273	0.60	0	(31)	0.22	1		R.MPCTEDYLSLILNR.L 2211 2212
2225	580.9391	1739.7955	1739.8222	-15.34	0	(36)	0.038	1		R.MPCTEDYLSLILNR.L 2226 2227 2228 2229 2230
2231	870.9153	1739.8161	1739.8222	-3.50	0	(60)	0.00024	1		R.MPCTEDYLSLILNR.L 2232
2233	874.3389	1746.6632	1746.6978	-19.80	0	43	0.0002	1		K.YNGVFQECCQAEDK.G 2234 2235 2236
2299	599.2803	1794.8191	1794.8247	-3.14	1	32	0.092	1	U	K.DDPHACYSTVFDKLK.H 2300 2301
2362	940.9627	1879.9108	1879.9138	-1.60	0	(9)	42	1	U	R.RPCFSALTPDETYVPK.A 2359 2360 2361
2372	627.6455	1879.9147	1879.9138	0.48	0	33	0.17	1	U	R.RPCFSALTPDETYVPK.A 2371 2373 2374 2375 2376 2377 2378 2379
2393	630.3128	1887.9166	1887.9195	-1.53	0	44	0.013	1		R.HPYFYAPELLYYANK.Y 2386 2387 2388 2389 2390 2391 2392 2394 2395
2405	634.6271	1900.8596	1900.8625	-1.55	1	9	18	1	U	R.NECFLSHKDDSPDLPK.L
2409	636.6452	1906.9137	1906.9135	0.12	0	32	0.19	1	U	K.LFTFHADICTLPDTEK.Q 2407 2408 2410 2411
2425	643.2751	1926.8036	1926.7910	6.54	1	35	0.0099	1	U	K.CCAADDKEACFAVEGPK.L
2473	673.9938	2018.9597	2018.9619	-1.09	1	(14)	10	1	U	K.LKPDPNTLCDEFKADEK.K 2472
2475	505.7482	2018.9638	2018.9619	0.93	1	14	9.8	1	U	K.LKPDPNTLCDEFKADEK.K 2474
2523	682.3379	2043.9918	2044.0206	-14.09	1	48	0.0046	1		R.RHPYFYAPELLYYANK.Y 1697 1698 2491 2492 2493 2494 2495 2496 2497 2
2536	512.0129	2044.0225	2044.0206	0.91	1	(35)	0.12	1		R.RHPYFYAPELLYYANK.Y 2530 2531 2532 2533 2534 2535 2537 2538 2539 2
2591	537.7728	2147.0622	2147.0568	2.50	2	5	1.3e+002	1	U	K.LKPDPNTLCDEFKADEKK.F 2590
2603	562.7425	2246.9409	2246.9354	2.45	1	13	1.6	1		K.ECCHGDLLECADDRADLAK.Y 2601 2602
2609	820.0647	2457.1724	2457.1733	-0.40	1	10	26	1	U	K.DAIPENLPPLTADFAEDKDVCK.N 2608
2636	829.7089	2486.1049	2486.1028	0.84	1	16	2.1	1		K.YNGVFQECCQAEDKGACLLPK.I 2629 2630 2632 2633 2634 2635
2646	1246.6340	2491.2534	2491.2570	-1.45	0	(11)	35	1	U	K.GLVLIAFSQYLQQCPFDEHVK.L 2647
2652	623.8212	2491.2556	2491.2570	-0.54	0	(21)	2.9	1	U	K.GLVLIAFSQYLQQCPFDEHVK.L 2649 2650 2653 2654
2657	831.4262	2491.2567	2491.2570	-0.11	0	48	0.007	1	U	K.GLVLIAFSQYLQQCPFDEHVK.L 2655 2658 2659 2660 2661 2662 2663
2740	523.2440	2611.1834	2611.1577	9.83	2	8	22	1		K.VHKECCHGDLLECADDRADLAK.Y
2813	878.6788	3510.6861	3510.6647	6.10	2	22	1.5	1	U	K.SHCIAEVEKDAIPENLPPLTADFAEDKDVCK.N 2810 2811 2812 2814 2815
2817	703.1450	3510.6886	3510.6647	6.81	2	(21)	1.7	1	U	K.SHCIAEVEKDAIPENLPPLTADFAEDKDVCK.N 2808 2809 2816

9. IL8_HUMAN **Mass:** 11376 **Score:** 80 **Matches:** 41(4) **Sequences:** 5(2) **emPAI:** 2.77
Interleukin-8 OS=Homo sapiens GN=CXCL8 PE=1 SV=1

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
997	416.2091	830.4037	830.4035	0.25	0	42	0.014	1	U	K.ENWVQR.V 991 992 993 994 995 996 998 999 1000 1001 1003 1004
1059	437.7206	873.4267	873.4266	0.09	0	47	0.0046	1	U	R.ELCLDPK.E 1057 1058 1060 1061 1062 1063
1455	368.8661	1103.5764	1103.5764	0.03	0	(15)	14	1	U	K.TYSKPFHPK.F 1450 1451 1453 1454 1457 1458 1459 1460
1462	552.7958	1103.5771	1103.5764	0.68	0	31	0.34	1	U	K.TYSKPFHPK.F 1463 1464 1465
1701	468.2388	1401.6946	1401.6922	1.72	1	6	82	5	U	K.LSDGRELCLDPK.E 1702
2252	883.9566	1765.8987	1765.9033	-2.61	0	(18)	5.9	1	U	R.VIESGPHCANTEIIVK.L 2251 2253
2254	589.6414	1765.9024	1765.9033	-0.47	0	28	0.6	1	U	R.VIESGPHCANTEIIVK.L 2255 2256

Supplementary information (SI) figure 16. 500 nM concentration, combined BSA & IL-8 digest 0.3µL injection volume

Mascot Search Results

```
User                  : mervyn lewis
Email                 : m.lewis@bath.ac.uk
Search title          :
MS data file          : 150630sample9.mgf
Database              : SwissProt 2015_07 (548872 sequences; 195617763 residues)
Timestamp             : 2 Jul 2015 at 09:28:10 GMT
Enzyme                : Trypsin
Fixed modifications   : Carbamidomethyl (C)
Variable modifications : Oxidation (M)
Mass values           : Monoisotopic
Protein Mass          : Unrestricted
Peptide Mass Tolerance : ± 20 ppm
Fragment Mass Tolerance: ± 0.1 Da
Max Missed Cleavages  : 2
Instrument type       : ESI-QUAD-TOF
Number of queries     : 1686
Protein hits          : ALBU_BOVIN Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4
                        ALBU_SHEEP Serum albumin OS=Ovis aries GN=ALB PE=1 SV=1
                        ALBU_FELCA Serum albumin OS=Felis catus GN=ALB PE=1 SV=1
                        ALBU_CANFA Serum albumin OS=Canis familiaris GN=ALB PE=1 SV=3
                        ALBU_MOUSE Serum albumin OS=Mus musculus GN=Alb PE=1 SV=3
                        ALBU_MACFA Serum albumin OS=Macaca fascicularis GN=ALB PE=2 SV=1
                        ALBU_HUMAN Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2
                        ENRN_BPT3  Endodeoxyribonuclease 1 OS=Enterobacteria phage T3 GN=3 PE=4 SV=1
                        ALBU_HORSE Serum albumin OS=Equus caballus GN=ALB PE=1 SV=1
```

Select Summary Report

Format As: Select Summary (protein hits) — Help

Significance threshold p< 0.05 — Max. number of hits AUTO

Standard scoring / MudPIT scoring / Ions score or expect cut-off 0 — Show sub-sets 0

Show pop-ups / Suppress pop-ups — Require bold red

Preferred taxonomy: All entries

Re-Search — All queries / Unassigned / Below homology threshold / Below identity threshold

1. ALBU_BOVIN **Mass:** 71244 **Score:** 2073 **Matches:** 360(54) **Sequences:** 43(16) **emPAI:** 4.06
Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
642	333.1918	664.3691	664.3697	-0.86	1	20	1.9	1		K.KFWGK.Y 641 644 646
684	379.7151	757.4156	757.4156	-0.08	0	46	0.0084	1		K.GACLLPK.I 681 682 683 685 686 687 688
695	395.2394	788.4643	788.4644	-0.14	0	22	2.3	1		K.LVTDLTK.V 691 692 693 697 698 700
706	424.2546	846.4946	846.4963	-2.09	1	19	3.6	2		R.LSQKFPK.A 704 705 708 709
814	449.7443	897.4740	897.4742	-0.20	0	8	32	2		R.LCVLHEK.T 815 816 817
834	461.7478	921.4810	921.4807	0.24	0	48	0.0061	1	U	K.AEFVEVTK.L 827 828 829 830 831 832 833 835 836 837 838
842	464.2498	926.4851	926.4861	-1.09	0	30	0.19	1		K.YLYEIAR.R 840 841 843 844 845 846 847 848 849 850 851 852 853 854 85
871	501.7944	1001.5742	1001.5757	-1.49	0	34	0.16	1	U	K.LVVSTQTALA.- 870 872 873 874 875 876 877 878 879 880 881 882 883
885	507.8132	1013.6118	1013.6121	-0.24	0	61	0.00024	1		K.QTALVELLK.H 886 887 888 889 890 891
1033	534.7213	1067.4280	1067.4342	-5.84	0	28	0.045	1	U	K.QNCDQFEK.L 1031 1034 1035 1036
1049	569.7522	1137.4898	1137.4907	-0.79	0	20	0.82	1		K.CCTESLVNR.R 1046 1047 1048
1055	571.8595	1141.7044	1141.7070	-2.27	1	43	0.01	1		K.KQTALVELLK.H 1050 1051 1052 1053 1054 1056 1057
1074	582.3179	1162.6213	1162.6234	-1.76	0	56	0.0012	1	U	K.LVNELTEFAK.T 1071 1072 1073 1075 1076 1077 1078 1079 1080
1100	417.2115	1248.6127	1248.6139	-0.94	1	22	2.3	1		R.FKDLGEEHFK.G 1094 1096 1097 1103
1116	653.3610	1304.7074	1304.7088	-1.11	0	38	0.063	1		K.HLVDEPQNLIK.Q 1107 1108 1109 1110 1111 1112 1113 1114 1115 1117 11
1132	700.3368	1398.6590	1398.6853	-18.86	0	67	3.9e-005	1		K.TVMENFVAFVDK.C 1133 1134 1135 1136 1137
1139	473.9019	1418.6839	1418.6864	-1.76	0	34	0.11	1		K.SLHTLFGDELCK.V 1138 1140 1141 1142 1143 1144 1145 1146 1147
1151	710.3503	1418.6861	1418.6864	-0.19	0	(32)	0.16	1		K.SLHTLFGDELCK.V 1148 1149 1150 1152
1162	480.6083	1438.8029	1438.8045	-1.06	1	42	0.016	1		R.RHPEYAVSVLLR.L 1154 1155 1156 1157 1158 1159 1160 1161 1163 1164 1
1173	722.3216	1442.6286	1442.6347	-4.25	0	49	0.00092	1	U	K.YICDNQDTISSK.L 1172 1174 1175 1176 1178 1179
1191	732.2946	1462.5747	1462.5817	-4.78	0	28	0.021	1	U	K.TCVADESHAGCEK.S 1190
1195	488.5341	1462.5805	1462.5817	-0.80	0	(16)	0.33	1	U	K.TCVADESHAGCEK.S 1192 1193 1194 1196 1197
1214	740.3984	1478.7822	1478.7881	-4.02	0	78	5.3e-006	1	U	K.LGEYGFQNALIVR.Y 1209 1210 1211 1212 1213 1215 1216
1240	751.8093	1501.6041	1501.6065	-1.59	0	63	8.3e-006	1	U	K.EYEATLEECCAK.D 1239 1241 1242
1253	511.5982	1531.7727	1531.7738	-0.73	1	4	1.6e+002	1		K.LKECCDKPLLEK.S
1270	777.8266	1553.6387	1553.6457	-4.47	0	(11)	2.1	1	U	K.DDPHACYSTVFDK.L
1274	518.8904	1553.6495	1553.6457	2.46	0	17	0.64	1	U	K.DDPHACYSTVFDK.L 1275 1277 1280
1285	784.3728	1566.7310	1566.7354	-2.83	0	85	6.7e-007	1	U	K.DAFLGSFLYEYSR.R 1281 1282 1283 1284 1286 1287 1288 1289 1290 1291 1
1301	526.2612	1575.7619	1575.7603	1.00	0	25	1.2	1	U	K.LKPDPNTLCDEFK.A 1293 1294 1296 1297 1298 1299 1300
1319	547.3171	1638.9293	1638.9305	-0.70	1	62	0.00013	1		R.KVPQVSTPTLVEVSR.S 1311 1312 1313 1314 1315 1316 1317 1318 1320 132
1341	862.9205	1723.8264	1723.8273	-0.50	0	72	1.6e-005	1		R.MPCTEDYLSLILNR.L 1342 1343 1344 1345 1346
1347	575.6165	1723.8276	1723.8273	0.15	0	(38)	0.049	1		R.MPCTEDYLSLILNR.L 1348 1349 1350 1351 1352
1353	580.9461	1739.8165	1739.8222	-3.29	0	(27)	0.42	1		R.MPCTEDYLSLILNR.L 1070 1354
1356	870.9171	1739.8197	1739.8222	-1.44	0	(41)	0.019	1		R.MPCTEDYLSLILNR.L 1355 1357
1359	874.3556	1746.6966	1746.6978	-0.67	0	23	0.075	1		K.YNGVFQECCQAEDK.G 1358 1360
1379	599.2809	1794.8208	1794.8247	-2.19	1	13	7.8	1	U	K.DDPHACYSTVFDKLK.H 1377 1380 1381
1397	627.6443	1879.9110	1879.9138	-1.50	0	22	2.3	1	U	R.RPCFSALTPDETYVPK.A 1390 1391 1392 1393 1394 1395 1396 1398 1399 14
1411	630.3251	1887.9534	1887.9195	17.9	0	37	0.093	1		R.HPYFYAPELLYYANK.Y 1402 1403 1404 1405 1406 1407 1408 1409 1410
1417	636.6459	1906.9158	1906.9135	1.18	0	29	0.41	1	U	K.LFTFHADICTLPDTEK.Q 1412 1413 1414 1415 1416
1424	643.2685	1926.7837	1926.7910	-3.77	1	16	0.41	1	U	K.CCAADDKEACFAVEGPK.L 1425 1426
1442	673.9935	2018.9586	2018.9619	-1.61	1	(7)	50	1	U	K.LKPDPNTLCDEFKADEK.K 1440 1443 1448 1449
1454	505.7471	2018.9595	2018.9619	-1.19	1	19	3.2	1	U	K.LKPDPNTLCDEFKADEK.K 1452 1457 1459
1491	682.3459	2044.0158	2044.0206	-2.36	1	27	0.73	1		R.RHPYFYAPELLYYANK.Y 1463 1464 1465 1466 1467 1468 1469 1470 1471 14
1495	512.0134	2044.0245	2044.0206	1.87	1	(8)	55	1		R.RHPYFYAPELLYYANK.Y 893
1529	562.7396	2246.9292	2246.9354	-2.78	1	14	0.92	1		K.ECCHGDLLECADDRADLAK.Y 1527 1530 1532
1534	749.9839	2246.9300	2246.9354	-2.41	1	(2)	15	1		K.ECCHGDLLECADDRADLAK.Y
1556	820.0653	2457.1740	2457.1733	0.29	1	6	61	1	U	K.DAIPENLPPLTADFAEDKDVCK.N 1555
1570	829.7073	2486.1002	2486.1028	-1.06	1	12	5.5	1		K.YNGVFQECCQAEDKGACLLPK.I 1571 1572 1573 1574 1575
1583	831.4237	2491.2492	2491.2570	-3.13	0	54	0.0017	1	U	K.GLVLIAFSQYLQQCPFDEHVK.L 1580 1581 1582 1584 1585 1586 1587 1588 1
1626	523.2389	2611.1579	2611.1577	0.06	2	2	47	1		K.VHKECCHGDLLECADDRADLAK.Y
1642	760.3202	3037.2515	3037.2416	3.27	1	1	12	2	U	K.EYEATLEECCAKDDPHACYSTVFDK.L
1647	703.1409	3510.6681	3510.6647	0.99	2	14	7.3	1	U	K.SHCIAEVEKDAIPENLPPLTADFAEDKDVCK.N 1648 1649 1650 1652 1653
1655	878.6753	3510.6720	3510.6647	2.08	2	(13)	11	1	U	K.SHCIAEVEKDAIPENLPPLTADFAEDKDVCK.N 1654 1656 1657 1658 1659 1660 1

Supplementary information (SI) figure 17. 500 nM concentration, BSA only digest 0.1µL injection volume

Conclusion

The aims of the research were broadly focused on how to use LC-MS/MS to expand our understanding of human and environmental health through the analysis of molecular biomarkers in river and wastewater. There were three primary themes to the research:

Aim one: To explore the uses of chirality in determining analyte origin and its impact on assessments of public and environmental health

Aim two: To develop new methodologies to maximise the information obtainable from currently utilised health biomarkers

Aim three: The exploration of proteins as potential new biomarkers of public health

Objective 1: To develop new methodologies to maximise the information obtainable from currently utilised health biomarkers and to explore the uses of chirality in determining analyte origin and its impact on assessments of public health by using *Wastewater-based epidemiology combined with local prescription analysis as a tool for temporal monitoring of drug trends – a UK perspective*

WBE was popularised by its ability to measure large populations quickly and relatively easily compared to more traditional survey-based approaches [1; 2]. However, governments and other large public health organisations have been slow to adopt WBE for use as anything more than a yearly snap shot of drug use in Europe. WBE can generate vast amounts of data about what is being consumed in a community and this can then be used to feedback to inform policy about the state of public health, which is difficult to estimate via traditional means. Five weekly periods of monitoring across five years in one city in the UK was examined to find trends in consumption that can provide information about the long-term state of DoA and pharmaceutical use in the UK. Trends in the consumption of other drugs of abuse was examined and placed into the wider context of European drug consumption, which showed that despite rising consumption of cocaine across Europe consumption in the UK was happening significantly faster. Meanwhile, MDMA consumption remained steady despite rising use across Europe. Chirality was also important here, as the detected EF of methamphetamine appeared to vary by year, suggesting a change in how it was being manufactured, which can directly support actions aimed at reducing the supply of methamphetamine. The backdrop to this research was initially the continued detection of mephedrone after being regulated, with the eventually reduction in use to < LOQ showing that UK drug policy can be effective. After 2016 the focus shifted to how the introduction of sweeping drug reform, courtesy of the 2016 psychoactive substance bill, changed drug consumption in the UK. This contextualisation of UK drug can then allow for decisions on drug policy to be taken in the appropriate context, which would then allow for appropriate response to tackle drug abuse in the UK.

The use of pharmaceuticals, particularly opioids and benzodiazepines, and a comparison to rates of prescription were also explored. Of particular interest was the relationship of prescription rates to wastewater concentration, which did not always agree. For compounds with low potential for abuse, like venlafaxine, there was good agreement between trends in wastewater and prescription. However prescription analysis could not fully explain use of drugs available without a prescription, like pseudoephedrine, or with potential for abuse or illicit supply like opioids.

Objective 2: To explore the uses of chirality in determining analyte origin and its impact on assessments of environmental health by determination of the *Stereochemistry of ephedrine and its environmental significance: Exposure and effects direct approach*.

The importance of chiral analysis was shown in chapter two by the changes in ephedrine stereochemistry observed and the impact this has on ephedrine ecotoxicity. In river water microcosms mimicking environmental conditions significant degradation only took place under biotic conditions, which showed that ephedrine degradation was primarily biological meaning it will be persistent in the environment until metabolised. The microcosms also showed how the synthetic ephedrines (1S,2R-(+)-ephedrine and 1R,2R-(-)-pseudoephedrine) were not degraded under any conditions, making them persistent in the environment and therefore a more potent micropollutant. Whereas under achiral chromatography all that would be observed was that the half-life of ephedrine was about two weeks.

Even when using single isomers chiral chromatography was crucial for detecting the formation of 1S,2R-(+)-ephedrine in river water microcosms containing only 1R,2S-(-)-ephedrine. This double chiral inversion was observed to take place after two weeks with the presence of any other isomer, which would be expected if it were formed via a non-enzymatic mechanism like elimination and nucleophilic addition.

Assessments of ephedrine toxicity using *D. magna*, *P. subcapitata* and *T. thermophile* showed that the synthetic ephedrines, generally, had much lower EC_{50} than their naturally occurring enantiomers. This combined with microcosm results showing the stability of the synthetic ephedrines, and the formation of 1S,2R-(+)-ephedrine from less toxic 1R,2S-(-)-ephedrine allowed for a better assessment of the ecotoxicity of ephedrine, by showing that a more toxic isomer could form under environmental conditions.

Objective 3: To develop new methodologies to maximise the information obtainable from currently utilised health biomarkers via *A multi-residue method by supercritical fluid chromatography coupled with tandem mass spectrometry method for the analysis of chiral and non-chiral chemicals of emerging concern in environmental samples*

The work presented so far has demonstrated that there was still a lot of roam for growth in how analytical analysis was undertaken and how data was used when trying to understand public and environmental health. To this end environmental analysis has fundamentally limited itself by not fully considering the importance micropollutant chirality has on environmental impact. SFC was shown to be an excellent technique for developing chiral methodologies that can track the progress of potential micropollutants through wastewater treatment and into the environment. SFC achieves this whilst maintaining still retaining the high sample throughput offered by reverse phase chromatography and required for routine analysis. In total 48-analytes, including five fully separated chiral molecules, were separated and analysed in only ten minutes. In comparison the same analytes were analysed using the same instrument in 35 minutes for achiral analysis [3] or 80 minutes for chiral analysis [4]. The method had excellent average limits of detecting and quantification, which were 16.7 and 46.7ng/L in river water and 20 and 52.3 ng/L in wastewater respectively. Whilst not quite at the fg L^{-1} level reported for some already regulated environmental pollutants [5] six analytes achieved a mLOQ $\leq$ 1.0 ng L^{-1}. This sensitivity is still sufficient to quantify 86% of analytes in at least one matrix with 50% being quantified in river water, effluent and influent including three chiral analytes. This is important as it would allow the method to monitor changes in EF as compounds pass through wastewater treatment in larger scale studies.

Whilst only five chirally separated analytes were successfully validated, the method demonstrated that for all its speed SFC was still able to give excellent separation of enantiomers. A range of alternative columns and mobile phases were trialled during method development and conditions were found to at least partially separate thirteen different chiral compounds, although the most separated fully by one method was five.

Objective 4: The exploration of proteins as potential new biomarkers of public health via *A new paradigm on public health assessment: water finger printing for protein markers of public health using mass spectrometry* and *Development of an enzymatic digestion method for use in wastewater for the analysis of proteins of disease by hydrophilic interaction liquid chromatography coupled to triple quadrupole mass spectrometry*

The rise of WBE for monitoring public health has been accompanied by a rise in the number of small molecule human health biomarkers examined, which now range from pharmaceuticals and drugs of abuse to NPS and biomarkers of oxidative stress as highlighted in chapter one. However, despite tentative steps towards monitoring human health using biomarkers of oxidative stress many current WBE biomarkers are primarily biomarkers of human consumption rather than human health. Urinary proteomics has the potential to revolutionise the field of UWF and WBE by introducing a huge range of potential human health biomarkers that are directly related to human health. Chapter five encompassed the development of a methodology for analysing proteins in wastewater using widely

available techniques and instrumentation with the goal of fitting proteomics into the established WBE workflow. The digestion procedure developed could be incorporated easily into current sample preparation procedures, taking only an additional two hours of analyst time to prepare samples with the majority of the preparation taking place overnight. The five proteins digested encompassed a wide range of molecular weights, from 8.4 kDa for IL-8 up to 66 kDa for BSA, which demonstrated that it was applicable to a wide range of proteins with varying amounts of cysteine-cysteine bonding to overcome. Whilst this diversity was beneficial in design the digest procedure the interleukins were not suitable first choice candidates for method development due to their low wastewater concentrations. The choice of limiting method development using the triple quadrupole to CRP and PSA was designed to help increase the methods ability to detect proteins in wastewater by focussing on only those most likely to be present at quantifiable concentrations. The observation of a protein of disease in wastewater is perhaps the most important outcome of this thesis as it opens up a whole new area of exploration in the quest for biomarkers of disease. In turn this method can be used as a baseline to help further refine the digestion method and increase its suitability for analysis in wastewater. In this way the process of digestion and analysis can be refined to create a method suitable for analysing proteins of disease for the purpose of monitoring public health.

Novelty and contribution statement

In order to effectively direct resources and guide policymakers there is an increasing need for accurate data on public and environmental health. Molecular diagnostics using LC-MS/MS is used in a number of ways to help gather such data, often through measurements of urban water. However, what was once an emerging field of research is becoming increasingly important in understanding and predicting public and environmental health, so it is vital that the data that is collected be of sufficient scope and quality for this purpose. This thesis has presented several novel approaches to highlight the need for better data and suggested ways it can be collected. Firstly, the innovative use of local prescription data for assessing drug consumption used in chapter one, which helped to give context to observed trends in wastewater concentrations and provided a means for assessing consumption of heroin. In chapter two, ephedrine was observed to both degrade and undergo chiral inversion under environmental conditions, which was important for confirming prior observations from wastewater treatment and for identifying the potential of CECs to convert into more toxic compounds *in-situ*. Chapter three addressed the lack of chiral methods of analysis for use in urban water by developing a method that could quantitatively analyse 95 CECs in less than 30 minutes using SFC. Chapter four's novelty came from the proposition that proteins could be used as a non-invasive method to measure public health through wastewater analysis. The chapter also identified several potential protein biomarkers, using a set of criteria that can be adapted by other researchers to identify other biomarkers. Finally, chapter five followed this by using established WBE procedures to estimate the

wastewater concentration of urinary biomarkers. Additionally, chapter five detailed the steps required to develop a bottom-up protein analysis method for use in wastewater and was able tentatively identify a peptide of C-reactive protein in wastewater for the first time.

Future work

Often during a PhD it feels as if no project is ever truly complete and this feeling extends even to published work. Chapter one contained a wealth of information and so there is likely a lot of future work around mining this data for further insights into public health, particularly examining the effect that prescription rates may have on pharmaceutical consumption beyond the simple links made in the chapter. The observation of increasing drug consumption from 2016 onwards should be followed up with more monitoring to see if this trend continues. In terms of whether this information can be used to inform policy future work could also be to investigate how to present data like this in a way that it can be digested by policy makers. Similarly, methods by which this data could be presented to the public could also be examined, with the goal of informing the public about how they can reduce the environmental impact of pharmaceuticals by curbing their own usage. Ultimately long term information about trends in drug use should be used to measure the efficacy of regulation on a broader scale than just country to country. The UK and Irish governments are in the fortunate position of being island nations, so drug policy that targets the supply of drugs can be simplified and limited to monitoring ports and shipping as their only land borders are with each other. Although in the wake of the UK's departure from the European Union the porosity of this border and the willingness of governments to cooperate on border policy and enforcement is currently up for debate. However, for continental Europe disrupting drug supply becomes much harder due to the presence of land borders and, mostly, free movement of people all over the continent, as well as land and sea borders to non-EU countries that sit astride important drug trafficking routes, such as Turkey and North Africa [6]. Long term data on drug use by country is useful for examining regional trends but this should be considered on a broader scale by organisations like the EMCDDA to look for broader geographic trends. This can then be used to propose drug policies on both a union and country level to reduce drug addiction. This already takes place to a degree but is focussed on changes in the type of cannabis used by region [7]. Ultimately legislation affecting the drug policy of individual countries is taken by that country's government, but if drug abuse is only considered on a local level then action taken may be misdirected or inadequate. The modular nature of drug policy within the EU also leads to country specific trends, such as the observed trend of cocaine usage in the UK increasing by more than the European average, which shows that whilst European wide action on cocaine may be required there are other UK specific reasons driving the increase within the country. Whether the UK and EU can cooperate on drug policy after Brexit is currently doubtful, with the UK at time of writing preparing to also leave Europol. Brexit will undoubtable affect the supply of drugs manufactured in Europe, both

licit and illicit, into the UK, particularly MDMA, which is mainly produced in the Netherlands and Belgium [7] and increasingly consumed in the UK and across continental Europe.

Similarly, the stereochemical fate of ephedrines in the environment could benefit from further research. The obvious first choice would be to repeat the microcosm experiment again and run them for a period longer than two weeks to observe how 1S,2R-(+)-ephedrine is formed and if given enough time 1R,2R-(-)-pseudoephedrine might also be produced, which would have a serious impact on the perceived ecotoxicity of ephedrine. Likewise, the ecotoxicity of mixtures of ephedrines could be trialled using other organisms like *D. Magna*, where only individual isomers have been studied, to look for synergistic effects, particularly at a ratio of ephedrine: pseudoephedrine that matches normal environmental conditions. This could then be expanded to examine a range of other CECs in order to understand their ecotoxicity with the ultimate goal of proving that environmental monitoring needs to consider chirality. With a more detailed appreciation of environmental toxicity researchers would be able to support the ability of governments and regulatory organisations to prioritise their resources towards tackling the most problematic CEC. The first WFD in 2000 [8] did a good job of identifying the most dangerous environmental pollutants and future work should look at either expanding this list of compounds or developing a further classification system for their environmental risk; similar to how drugs in the UK are categorised (nominally) by risk. It is discussed in chapter four that diclofenac and synthetic oestrogens were considered as additions to the WFD list of banned substances [5] due to their environmental toxicity but were not added. Ultimately this can be seen as a compromise between protecting future environmental and current human health. If compounds like synthetic oestrogens, or painkillers like ibuprofen that are present in high concentration in the environment already, cannot be banned under a tiered system they could at least be controlled appropriately. To achieve this tiered system of risk environmental risk assessments that consider chirality would need to be implemented for all currently registered active pharmaceutical ingredients (APIs) and excipients/additives. For many APIs this process would require government action as where generic formulations exist trying to decide who does the testing would be difficult, although for patented formulations the burden would lie with the manufacturer within the current regulatory framework. Additionally illicit drugs are generally poorly studied by ecotoxicity, despite being present in wastewater at concentrations greater than some licit pharmaceuticals. Despite many of these substances once being used as APIs the burden of testing them would likely fall on the state rather than their initial manufacturer's. Finally, unlike the current ERA system where ERAs cannot be used to deny regulation of an API a new system should be able to deny regulation of particularly environmentally dangerous APIs or formulations from entering the market and hence the environment.

Chapter three has perhaps the most to gain from future work, although the method itself was successfully validated. Once it no longer became possible to analyse all compounds via the same MS

method the SFC method should have been altered to allow for better separation and analysis of more biomarkers, instead of creating a one size fits all SFC method for three MS methods. By separating the betablockers from the drugs of abuse it may be possible to create a method for chirally separating each of them using the CEL-1 and AMY-1 column's respectively. However, recoveries were a large source of problems during method validation and an alternative approach could be considered to help determine why this was so problematic. Whilst the normal approach to poor recoveries is to select a new internal standard, many analytes that used a deuterated or ^{13}C version of the analyte as their internal standard still exhibited poor recoveries. Instead of comparing analyte concentrations spiked into matrix before SPE, to analyte concentrations in mobile phase it would maybe be fairer to compare them to analyte concentration in samples where analytes are added after SPE, which are currently used to calculate signal suppression. However the ultimate goal is not to create one method or three methods to analyse CEC but to inspire other researchers to create their own chiral SFC methods. The scale of the problem of analysing what is going into the environment is too big to be performed by any individual country or organisation and this means that researchers need to band together and analyse environment samples in the same way that drugs of abuse are analysed in chapter three by the SCORE network. The advantage of using SFC instead of chiral LC or, worse, achiral LC is that it is not currently very widely used and so standards can be set early in adoption. Meanwhile for SCORE each contributor performs analysis using their own instruments and methodologies, despite a common protocol on how samples should be collected [9], although a strict inter-lab validation procedure takes place each year to assess the suitability of these methods. This could complement the wide spreading environmental toxicity testing suggested earlier and can help guide prioritisation of CEC by observing changes in enantiomeric fraction occurring during wastewater, which suggests stereospecific metabolism.

Whilst chapter four is largely exempt from future work given its purpose was to be an introduction to the concept of combining WBE and urinary proteomics there are some areas where it could be further developed. A more in-depth discussion surrounding the estimation of wastewater concentrations of urinary proteins, as was discussed in chapter five, would allow for other researchers to consider whether to explore other potential biomarkers of disease and not just those discussed. It would also allow for a dialogue surrounding whether such estimations are appropriate, given that the assumption of a completely health population is obviously false but served to hopefully provide a baseline for future analysis.

Chapter five presented a streamlined version of enzyme method development and future work could explore other avenues that were ignored during initial development, such as the use of surfactants. Whilst sodium deoxycholate (SDC) was trialled it proved difficult to remove from digests without filtering. At the time this was problematic as relatively low volumes (<2 mL) were being used and it was hoped SPE would be sufficient to remove SDC from digests. Now that larger volumes of

wastewater are being digested filtration has become a necessity so SDC may be viable once again. Likewise, with a method developed in chapter six it would be possible to more easily refine the digestion process quickly without waiting for samples to be analysed by third parties. An alternative to the digestion procedure that could help increase sensitivity in wastewater would be to extract proteins from wastewater first and then digest them second. This would remove the need to scale up digests for larger volumes and would allow for the use of extraction techniques that focus on the unique properties of proteins. If the volume of reagents used was further scaled down to less than 1 mL it would also be possible for samples to be analysed immediately after digestion. The initial step towards trying to validate a method for the detection of proteins of disease in wastewater and as such there are several areas of future work that should be explored. The first is the introduction of an internal standard, such as a deuterated form of the CRP peptide GYS-K that was repeatedly detected in both buffer and wastewater with several peaks where only one would be expected. The introduction of an internal standard would also allow for recovery and other required pieces of method validation to be carried out. BSA should still be considered as a measure of digest efficiency and this can then be quantified against an internal standard rather than against an external calibration curve. The ultimate goal of this research would be to analyse proteins of infectious disease to provide an early warning system for epidemics and other seasonal outbreaks like influenza. The first step to achieving this would be to identify proteins of infectious disease, or proteins excreted in response to infectious disease, in urine. This would require large scale testing of infectious populations and would be extremely challenging, particularly for viruses like influenza that vary from strain to strain as this would require the identification of biomarkers that are common to each strain. Once identified proteins of infectious disease would need to be detected in wastewater and quantified. This would require a more refined version of the currently developed method to facilitate the detection of infectious disease at low concentrations to maximise its utility as an early warning system. For non-infectious disease future developments should focus either on non-specific biomarkers of public health, like CRP, rather than specific diseases like PSA and prostate cancer. A more general health marker can serve as its own measure of community health and can help direct interventions to the most needy areas instead of taking a once size fits all approach. This would allow for proteomics to be used to measure qualitatively or quantitatively the efficacy of measures undertaken to improve public health i.e. if measures to reduce cardiovascular disease were introduced their efficacy could be measured by a long term decline in the concentration of CRP [10]. The most pressing limitation in the quest for biomarkers of disease is a validated method to analyse them in wastewater. Once this has been achieved then the only limiting factor is deciding what proteins to analyse and how best to use the information obtained to inform and interpret public health.

One option would be to try and directly link protein biomarkers with small molecule WBE, similar to how biomarkers of oxidative stress (a relatively new class of biomarker in WBE) were linked to

smoking through analysis of well-established biomarkers such as nicotine and cotinine. For example, the concentration of CRP could be linked to wastewater concentrations of beta-blockers and other drugs of cardiovascular health, perhaps incorporating the prescription analysis detailed in chapter one. However, for this technology to be useful it must be applicable, and the difficulty of any public health monitoring is knowing how the data relates to what is being measured and when to intervene. Whilst simplistically we may want disease biomarker concentrations to go down, although as uromodulin shows this may not always be because of decreasing rates of disease [11; 12], what this means in reality can be difficult to quantify. Concentrations of heroin were observed to decrease in chapter one, which agreed with other epidemiological data [13] but does this mean that current UK drug policy is a success? Does it mean the UK is right to not have a needle exchange programme like other European countries? And what does it tell us about those continuing to use heroin? Likewise, if wastewater concentrations of CRP did not correlate with wastewater and prescribed concentrations of beta-blockers or with hospital data on cardiovascular disease does this mean intervention is necessary? The answer to all these questions is sadly that more research is needed to quantify if, when and where intervention is necessary. Despite this WBE is still a useful tool for increasing our understanding of human and public health even if we perhaps do not fully understand how to use what we now know.

Research outputs

During my PhD I had the opportunity to speak and present at a number of conferences and be involved with the organisation of others. I also contributed to several published articles not submitted as part of my thesis.

Conferences and symposia:

SCORE second training school: April 2015, Castellon, Spain – Attendee

Chemistry department symposia: May 2015 – Poster presentation

Testing the waters 2015: Second international conference on wastewater-based drug epidemiology: October 2015, Ascona, Switzerland – Oral presentation

Presentation to department: Spring 2016 – Oral presentation

Chemistry department symposia: May 2016 - Attendee

Emerging Analytical Professionals 2016: Analytical alchemy – Tablets, toxins and tonics: May 2016, Fareham, UK - Poster and oral presentation

- Best poster pitch prize winner “Quantitative proteomics for molecular diagnostics of public health”

Bright Spark Symposium 2016: September 2016, University of Warwick, UK – Poster presentation

Wetskills Water Challenge programme: April 2017, University of Bath, UK – Organising committee

18th Young Water Professionals conference: April 2017, University of Bath, UK – Organising committee and workshop facilitator

Bolland symposium (department event) 2017: May 2017 – Oral presentation

Emerging Analytical Professionals 2017: The theory of everything – Launching the careers of analytical scientists: May 2017, Kettering, UK – Organising committee, session chair and technical presentation

SETAC UK annual meeting: August 2017, Oxford, UK – Poster presentation

Bright Spark Symposium 2017: September 2017, University of Bath, UK – Conference Chair and organiser

Testing the waters 2017: 3rd international conference on wastewater-based epidemiology: October 2017, Lisbon, Portugal – Oral presentation "Quantitative proteomics for molecular diagnostics of public health"

- Best oral presentation prize winner

Hyphenated techniques in chromatography (HTC-15): January 2018, Cardiff, UK – Oral presentation

Emerging Analytical Professionals 2018: The circle of life – Analysing the world around us: May 2018. Knutsford, UK – Organising committee, session chair

Bright Spark Symposium 2018: September 2018, University of Manchester, UK – Organising committee and session chair

Emerging Analytical Professionals 2019: Exploring the analytical sciences: May 2019, Leeds, UK – Organising committee and session chair

Emerging Analytical Professionals 2020: May 2020, Kettering, UK – Organising committee and conference vice-chair

Publications:

Sims, N., J. Rice, and B. Kasprzyk-Hordern. *An ultra-high-performance liquid chromatography tandem mass spectrometry method for oxidative stress biomarker analysis in wastewater* (2019) Anal. Bioanal. Chem. Volume 411, issue 11, pp 2261-2271

Camacho-Munoz, D., B. Petrie, L. Lopardo, K. Proctor, J. Rice, J. Youdan, R. Barden and B. Kasprzyk-Hordern. *Stereoisomeric profiling of chiral pharmaceutically active compounds in wastewaters and the receiving environment – A catchment-scale and a laboratory study* (2019) Environ. Int. Volume 127, pp 558-572

Other activities:

Associate member of the royal society of chemistry (RSC): 2015-

Alan Tetlow bursary winner – 2015: Funding to attend testing the waters 2017 conference. Article published in Water Science Forum (WSF) newsletter volume 16 issue 2.

Member of the analytical science network (ASN): 2016-

SETAC member: 2017-2018

ASN website coordinator: 2017-

ASN representative to the analytical division council of the RSC: 2018-

References:

[1] Daughton, C.G., 2001. Emerging pollutants, and communicating the science of environmental chemistry and mass spectrometry: Pharmaceuticals in the environment. *Journal of the American Society for Mass Spectrometry,* 12(10), pp. 1067-1076.
[2] Zuccato, E., Chiabrando, C., Castiglioni, S., Calamari, D., Bagnati, R., Schiarea, S. and Fanelli, R., 2005. Cocaine in surface waters: a new evidence-based tool to monitor community drug abuse. *Environmental health : a global access science source,* 4, pp. 14-14.
[3] Petrie, B., Youdan, J., Barden, R. and Kasprzyk-Hordern, B., 2016. Multi-residue analysis of 90 emerging contaminants in liquid and solid environmental matrices by ultra-high-performance liquid chromatography tandem mass spectrometry. *Journal of Chromatography A,* 1431, pp. 64-78.
[4] Evans, S.E., Davies, P., Lubben, A., and Kasprzyk-Hordern, B., 2015. Determination of chiral pharmaceuticals and illicit drugs in wastewater and sludge using microwave assisted extraction, solid-phase extraction and chiral liquid chromatography coupled with tandem mass spectrometry. *Analytica Chimica Acta,* 882, pp. 112-126.
[5] Loos, R., 2012. Analytical methods relevant to the European Commission's 2012 proposal on Priority Substances under the Water Framework Directive. JRC scientific and policy reports: Luxembourg: Publications office of the European Union, 2012.
[6] European Monitoring Centre for Drugs and Drug Addiction and Europol, 2019. *EU drug markets report 2019.* Luxembourg: Publications office of the European Union.
[7] EMCDDA, E.M.C.f.D.a.D.A., 2019. *European Drug report 2019: Trends and developments.* Luxembourg: Publications Office of the European Union.
[8] European Commission, 2002. *Implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results.* Official Journal of the European Communities.
[9] Castiglioni, S., Bijlsma, L., Covaci, A., Emke, E., Harman, C., Hernandez, F., Kasprzyk-Hordern, B., Ort, C., van Nujis, A.L.N., de Voogt, P. and Zuccato, E., 2016. Estimating community drug use through wastewater-based epidemiology. *Assessing illict drugs in wastewater: advances in wastewaster-based epidemiology.* Publications office of the European Union, Luxembourg, pp. 17-34.
[10] Kuhn, E., Wu, J., Karl, J., Liao, H., Zolg, W. and Guild, B., 2004. Quantification of C-reactive protein in the serum of patients with rheumatoid arthritis using multiple reaction monitoring mass spectrometry and C-13-labeled peptide standards. *Proteomics,* 4(4), pp. 1175-1186.
[11] Hammond, T.G., Moes, S., Youhanna, S., Jennings, P., Devuyst, O., Odermatt, A. and Jeno, P., 2016. Development and characterization of a pseudo multiple reaction monitoring method for the quantification of human uromodulin in urine. *Bioanalysis,* 8(12), pp. 1279-1296.
[12] Rampoldi, L., Scolari, F., Amoroso, A., Ghiggeri, G. and Devuyst, O., 2011. The rediscovery of uromodulin (Tamm-Horsfall protein): from tubulointerstitial nephropathy to chronic kidney disease. *Kidney International,* 80(4), pp. 338-347.
[13] EMCDDA, 2018. *United Kingdom Country Drug Report 2018.* Available from: *www.emcdda.europa.eu/countries/drug-reports/2018/united-kingdom*.